FINAL REPORT

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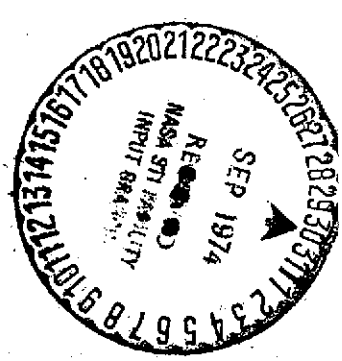
CASSETTE BACTERIA DETECTION SYSTEM

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
LYNDON B. JOHNSON SPACE CENTER
HOUSTON, TEXAS 77058

Contract No. NAS 9-13256

1 August 1974



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15 April 1974

C. B. Linnecke
Project Engineer

W. H. Hartung, Manager
Engineering Operations

AEROJET MEDICAL AND BIOLOGICAL SYSTEMS
9200 East Flair Drive
El Monte, California 91734

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1.0 SUMMARY

The design, fabrication, and testing of an automatic bacteria detection system with a zero-g capability and based on the filter-capsule approach has been completed. This system is intended for monitoring the sterility of regenerated water in a spacecraft.

The principle of detection is based on measuring the increase in chemiluminescence produced by the action of bacterial porphyrins (i.e., catalase, cytochromes, etc.) on a luminol-hydrogen peroxide mixture. Since viable as well as nonviable organisms initiate this luminescence, viable organisms are detected by comparing the signal of an incubated water sample with an unincubated control. High signals for the former indicate the presence of viable organisms.

System features include disposable sterile capsules (each containing a filter membrane) for processing discrete water samples, a transporter system for moving capsules through the processing sequence for addition of nutrient, incubation, four molar urea wash, and reaction of luminol-hydrogen peroxide in front of a photomultiplier tube. Liquids stored in pressurized containers are pumped by air-operated piston pumps to a needle which pierces a rubber septum in the base of the capsule.

Sensitivity goals of the instrument (< 5 cells/ml viable, < 10 cells/ml total, both for 400-ml sample) have been exceeded. Tests were conducted at challenges of 4×10^3 E. coli total cycle (10 cells/ml for 400-ml sample) and 2×10^3 E. coli viable cycles (5 cells/ml for 400-ml sample) and were reliably demonstrated. Challenges of 8×10^2 E. coli total cycle (2 cells/ml for 400-ml sample) revealed significant increases in signal. Background levels of the system varied significantly due to nutrient/system contamination and/or water contamination, causing signal levels to vary; however, challenges of 2 to 4×10^3 E. coli were always detectable.

Difficulties were encountered in the program when a Viton bladder for the nutrient supply failed during the sterilization procedure. An ethylene propylene bladder has been recommended as a replacement to allow wet sterilization. Further testing is necessary with the integrated system to

verify that the nutrient supply subsystem can be sterilized. If the bladder functions as expected, all design goals of this program will have been achieved.

2.0 INTRODUCTION

This is the final report submitted in accordance with the requirements of Contract NAS 9-13256, "Cassette Bacteria Detection System."

The objective of this contract was to develop a fully automatic chemiluminescence bacteria detection system based on the filter-capsule approach. This system is intended to provide "real-time" monitoring of the sterility of reclaimed water in a space environment. The present effort was designed to upgrade a preprototype fully functional unit with laboratory containers for reagent storage to a prototype unit with zero-g compatible tankage.

The principle of detection is based on measuring the increase in chemiluminescence produced by the reaction of bacterial porphyrins with a luminol-hydrogen peroxide mixture. To permit differentiation between living and dead organisms, integrated chemiluminescent signals are obtained for both incubated and unincubated bacterial samples. A higher signal for the incubated sample indicates the presence of viable organisms.

The system fabricated under the present program utilizes disposable filter capsules for processing the individual water samples. Filter capsules, each containing a sterile membrane filter, are dispensed automatically from a cassette onto a continuous chain transport which conveys the capsule from one station to another. Liquids are introduced by means of a hypodermic needle which pierces a rubber septum in the bottom of the capsule. During the processing cycle, discrete volumes of the respective reagents are dispensed by syringe pumps from pressurized storage tanks. The processing sequence involves sample filtration, nutrient addition, incubation, washing with 4 M Urea and reaction with luminol-hydrogen peroxide reagent at the readout station. The intensity of the luminescence produced on reaction is monitored by a photomultiplier tube with the processed signal displayed on a strip chart recorder.

The system developed under the present contract and the results of the evaluation of this instrument are described in detail below.

3.0 EXPERIMENTAL RESULTS

3.1 INSTRUMENTATION

3.1.1 Filter Capsule

Illustrations of the capsule used for sample processing is shown in Figure 1.

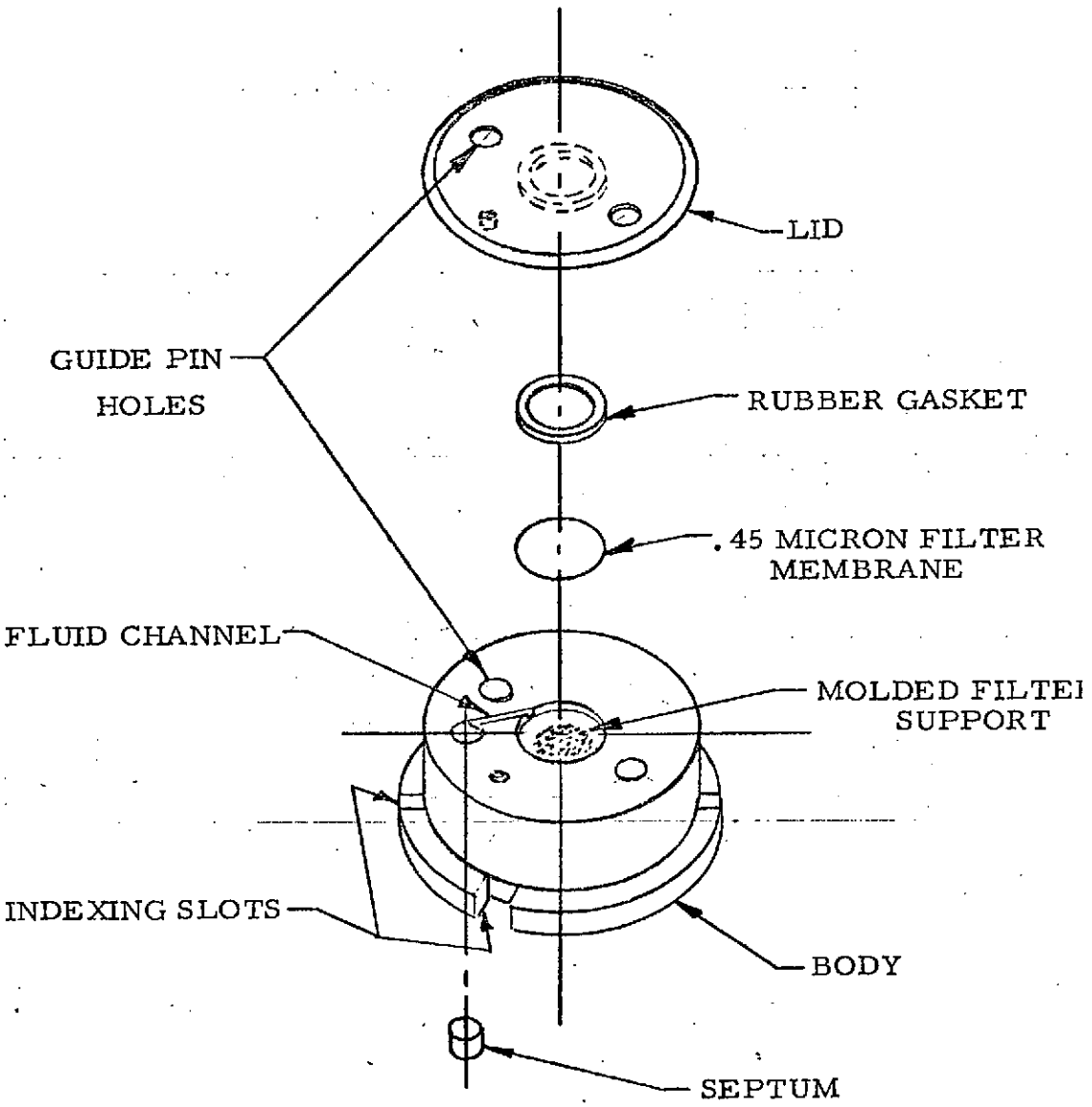
The two-piece capsule, molded out of Plexiglas (polymethyl methacrylate), consists of a clear lid cemented to a black pigmented body. The latter contains a perforated grid in the center for supporting the filter membrane. Incorporated in the lid are (1) a ring which provides a 50% compression on the filter and rubber gasket, and (2) a pin for aligning the lid with the body on assembly. Guide pin holes in the body of the capsule assure proper station alignment. Liquids are introduced by a syringe needle through a rubber septum located near the periphery of the capsule body. Solid-pointed needles with holes on the side are used at all of the stations to minimize plugging of the needle. The liquid is transported to the filter area by a connecting channel.

When the plunger engages the bottom of the capsule, the O-ring on the plunger bears against a 45° chamfer on the capsule bottom.

In assembling the capsule a special adhesive is applied to the capsule body, the filter and gasket dropped into position, and the lid aligned with the body of the capsule. The capsule is then placed under compression in a spring clamp assembly (Figure 2) for two hours. The assembled capsule is then post-cured overnight at $37^{\circ}C$ to insure a complete set of the adhesive. The capsule is then checked for leakage or plugging by hand injecting about 2 ml of 4 M Urea. Further post-treatment with luminol-hydrogen peroxide is used to insure removal of trace impurities which might produce a spurious signal. The capsule is then flushed with filtered distilled water, the residual water blown out with filtered air and the capsule then placed in a (P_2O_5) desiccator for final drying. The capsule is then ready for use. A detailed procedure for treating and assembling the capsule is given in Appendix E.

The development studies leading to the present capsule design are described in Section 4.0, Laboratory Support Studies.

Figure 1 Capsule, Exploded View



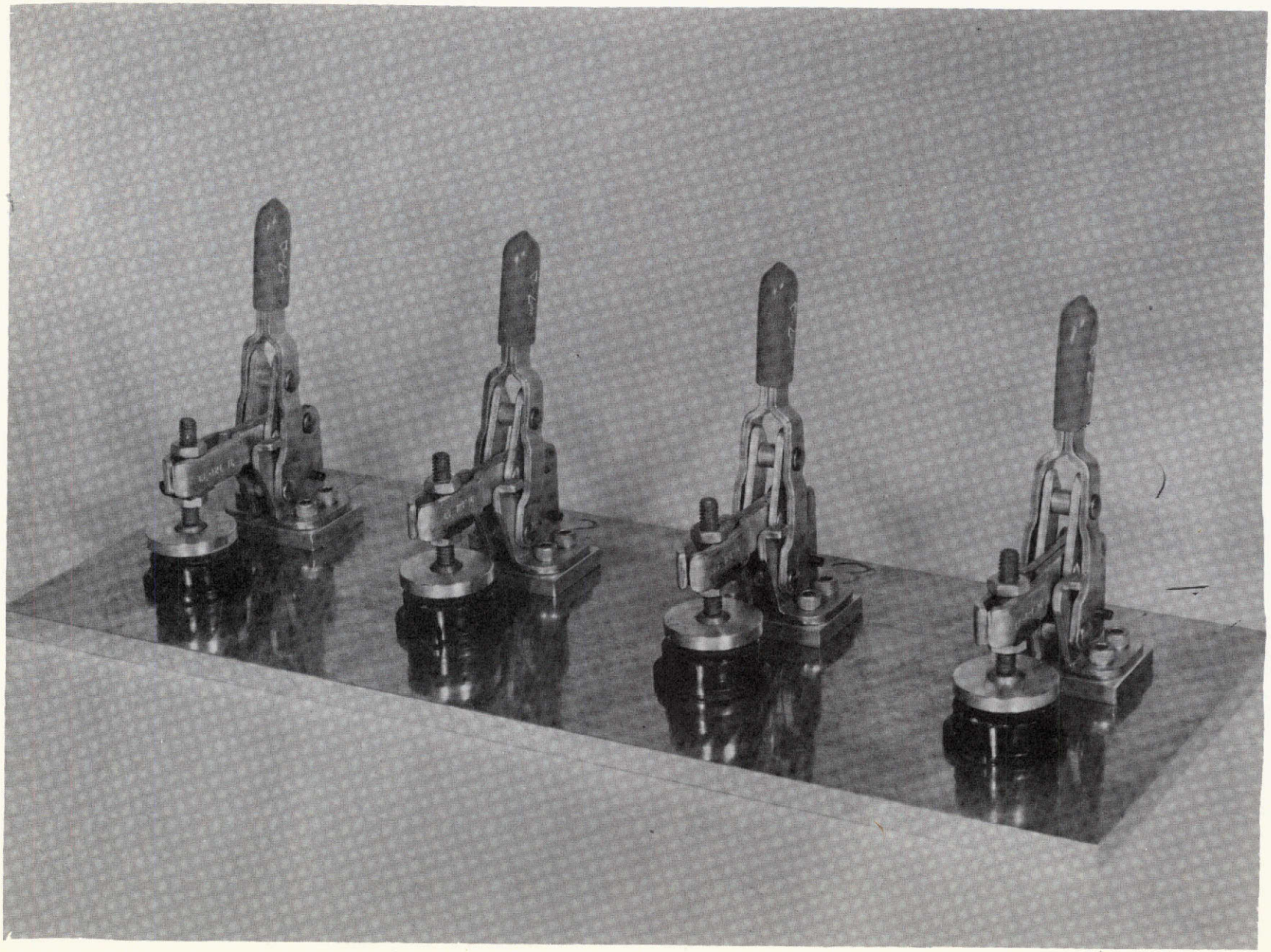


Figure 2 Capsule Clamping Fixture

3.1.2 Integrated Detection System

The Cassette Bacteria Detection System shown in Figures 3 through 7 contain three major assemblies integrated into a single unit. These consist of (1) a sample processing assembly (Figure 3), (2) a reagent storage and pump metering system (Figures 4 through 6) and (3) an electronic readout and control assembly (Figure 7). A brief description of each of these is given below.

a. Sample Processing Assembly

The functions of this assembly are to concentrate a water sample by pressure filtration through the capsule and then to react the deposited organisms with reagent in front of a photomultiplier tube. This subsystem, shown in Figures 3 and 4, contains a load station for loading the capsules on to a chain drive transport, individual sample processing stations (i.e., sample concentration, nutrient addition, incubator, urea wash, reaction and readout), and an unload station for removing the capsule from the conveyor. Except for the incubator each processing station has an inlet line which leads to the syringe needle and an outlet line which exhausts to the waste pump.

The capsule is conveyed from station-to-station on a metal plate supported between two roller chains (Figure 3). Indexing of the capsule is accomplished by means of a sprocket arrangement driven by an adjustable rack and pinion.

A more detailed description of the various stations is given below. The liquid flow schematic is contained in Figure 8.

(1) Capsule Load Station

Capsules are loaded on to the carrier or support from underneath by a piston arrangement (i.e., pressurized air is used to drive a Teflon piston behind the capsules stacked in the loaded cylinder). To insure proper alignment, the piston and capsules are slotted at the periphery to engage a guide rail mounted in the cylinder. Two spring-loaded studs, located at the base of the cylinder, retain the capsules until loading is required. A small air cylinder on top of the station acts as a guide to prevent cocking on

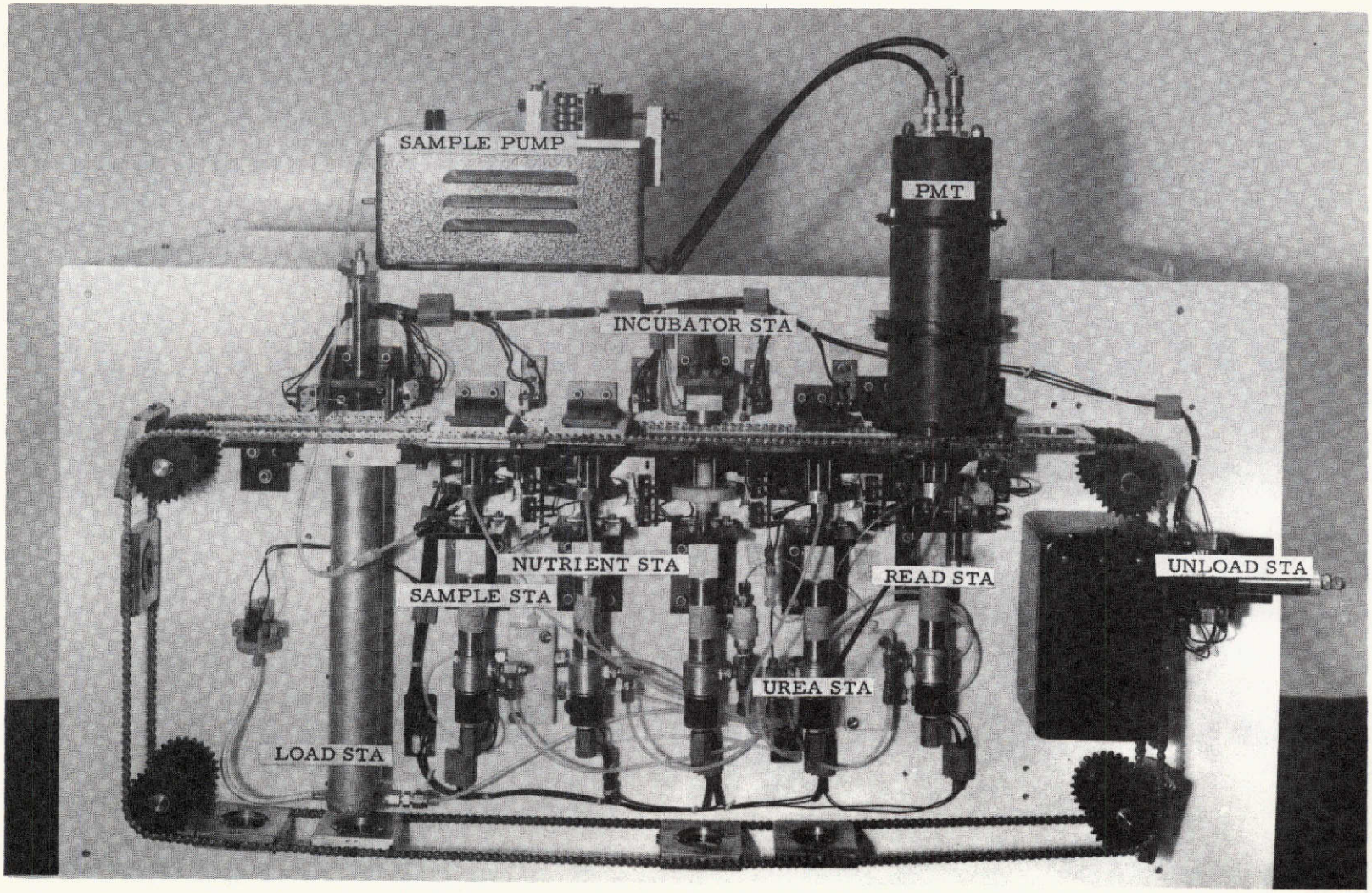


Figure 3 System Front View

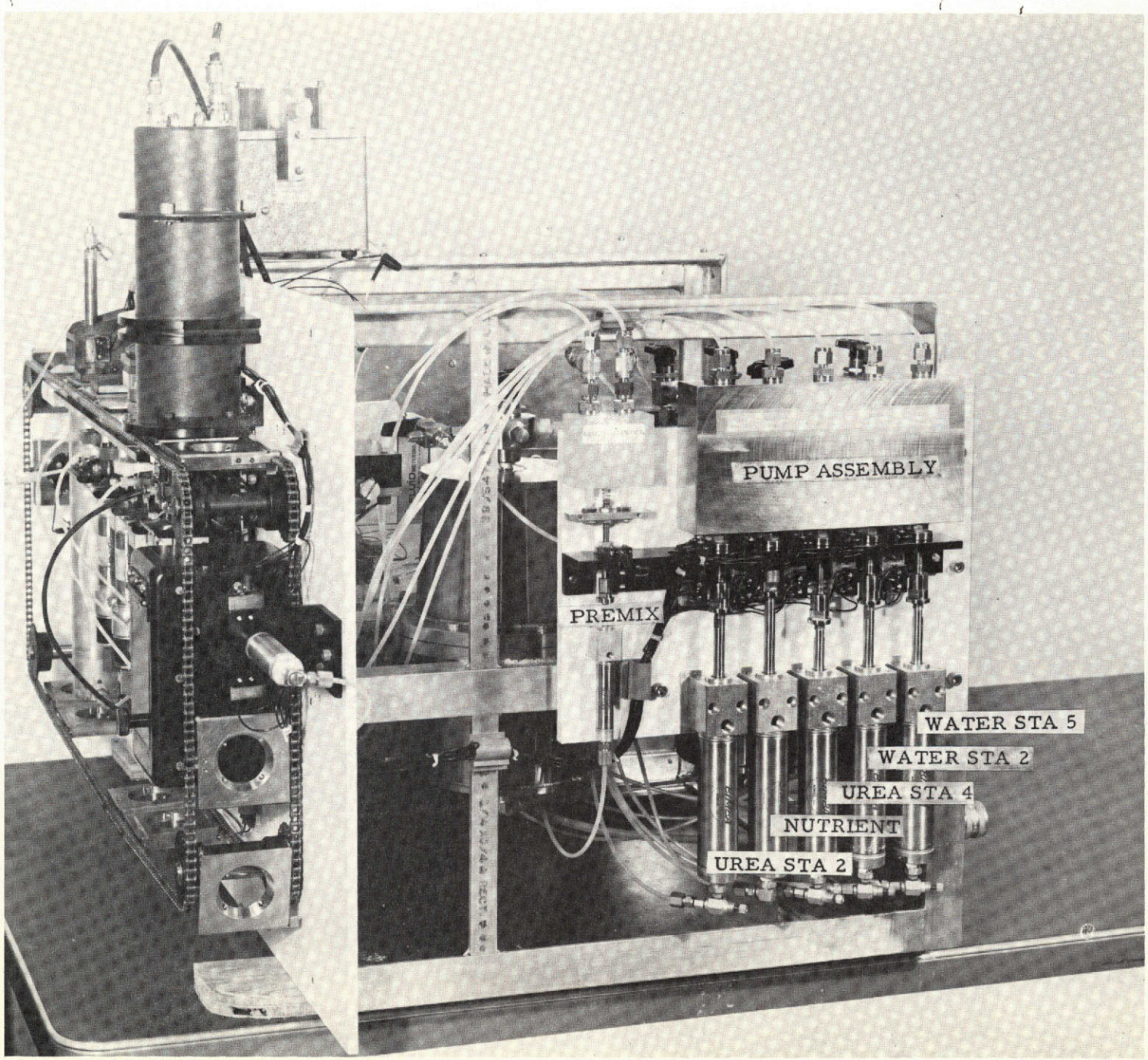


Figure 4 Reagent Pump Assembly

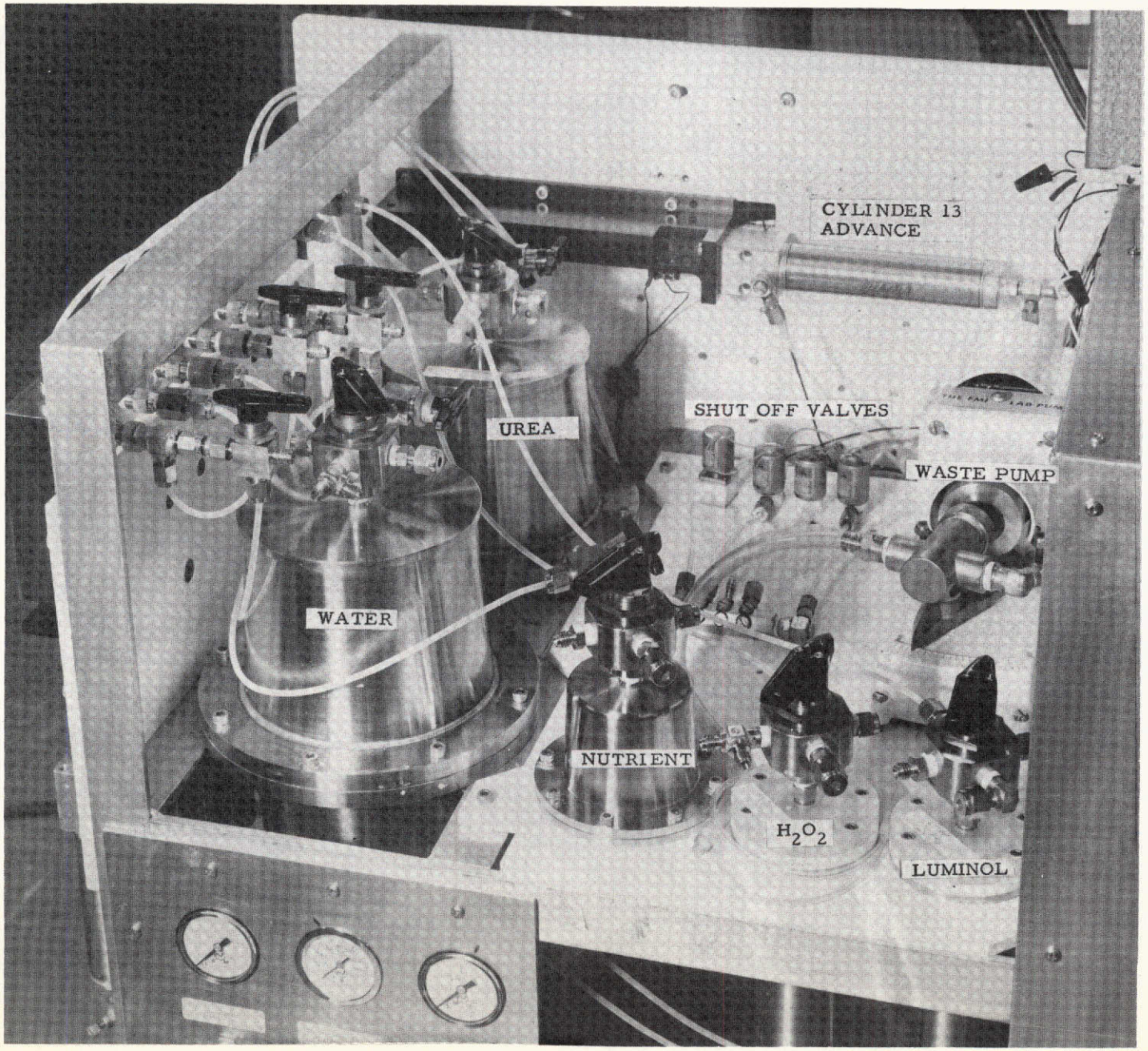


Figure 5 Reagent Containers

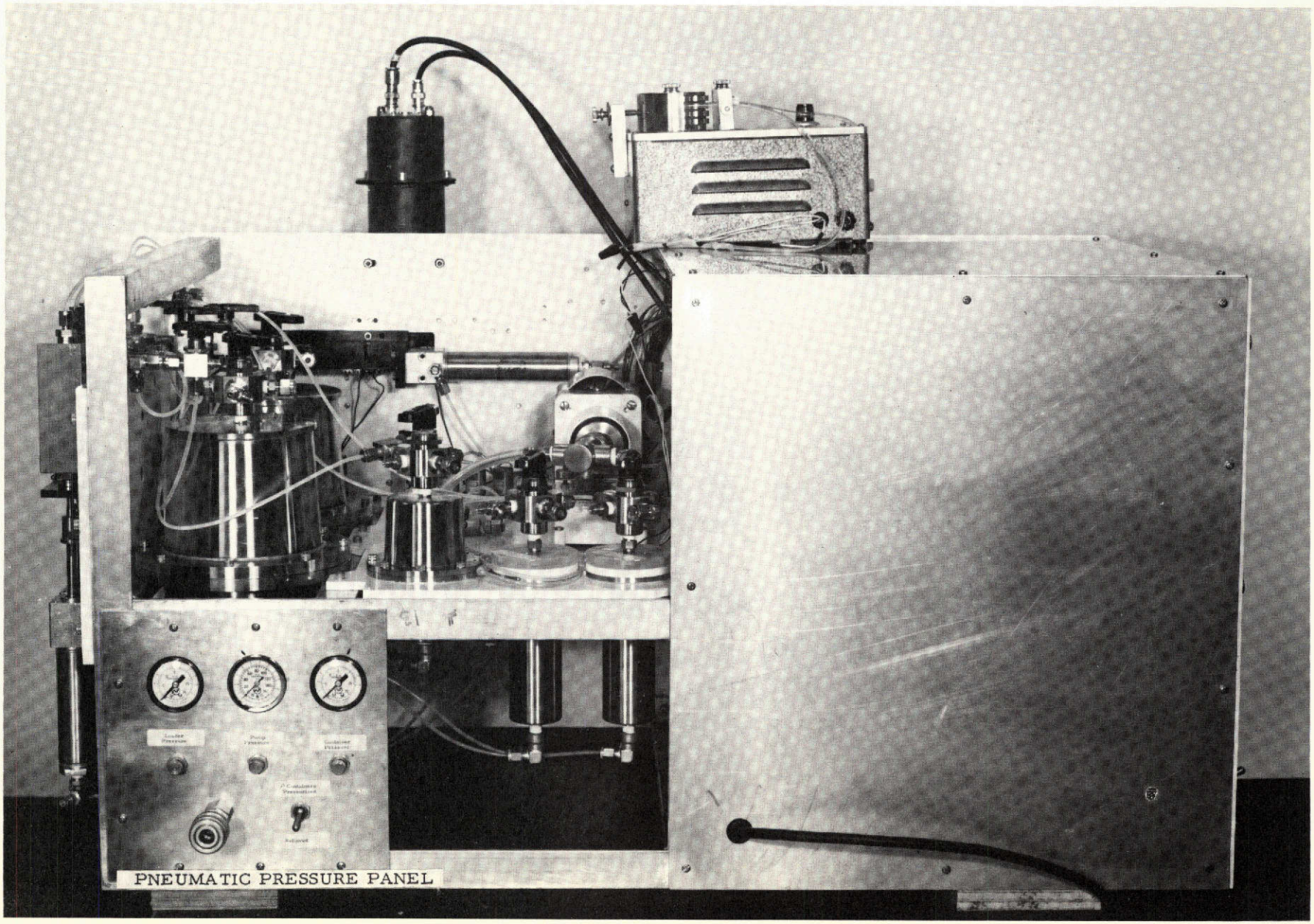


Figure 6 System Rear View

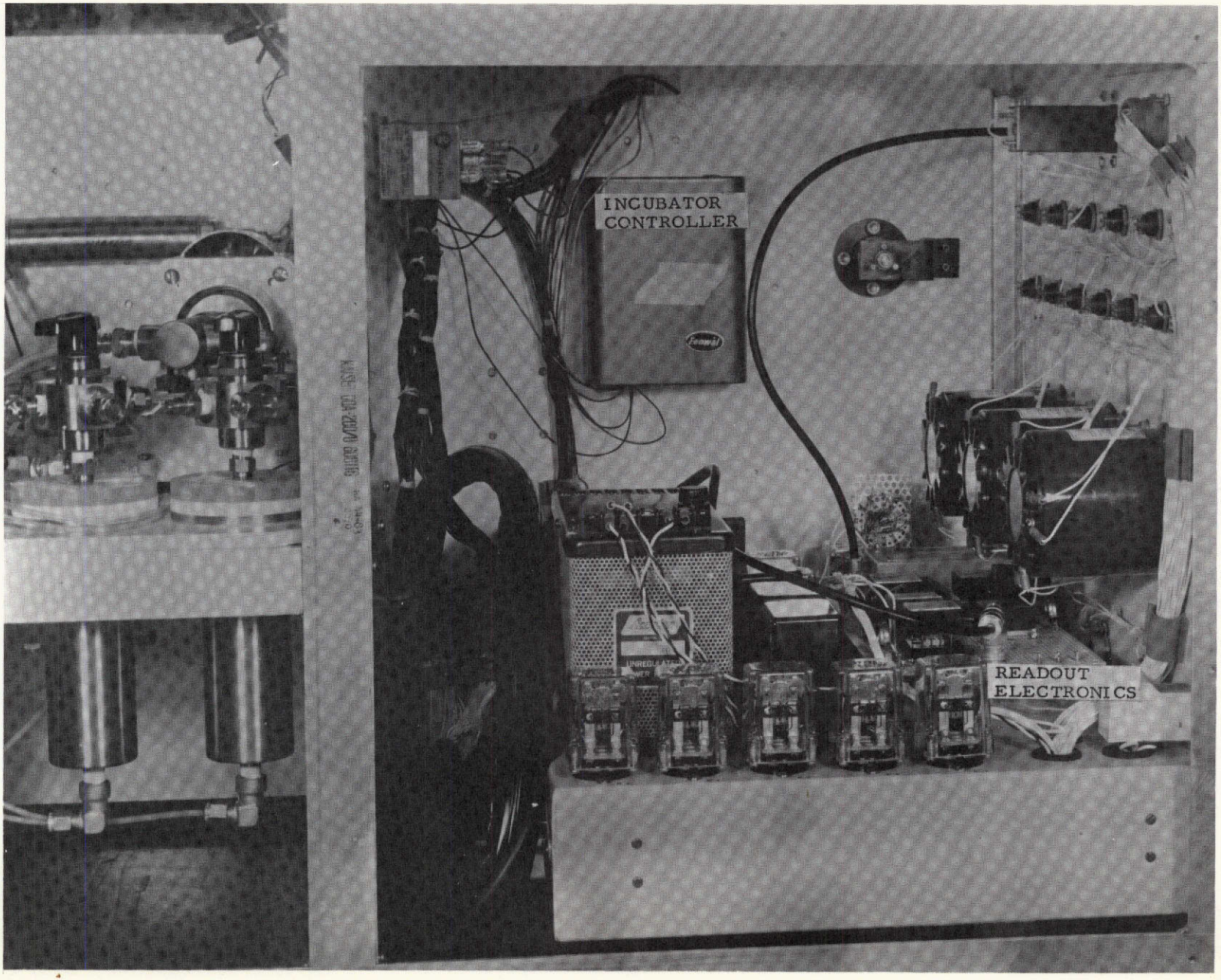


Figure 7 Electronic Readout and Control Assembly

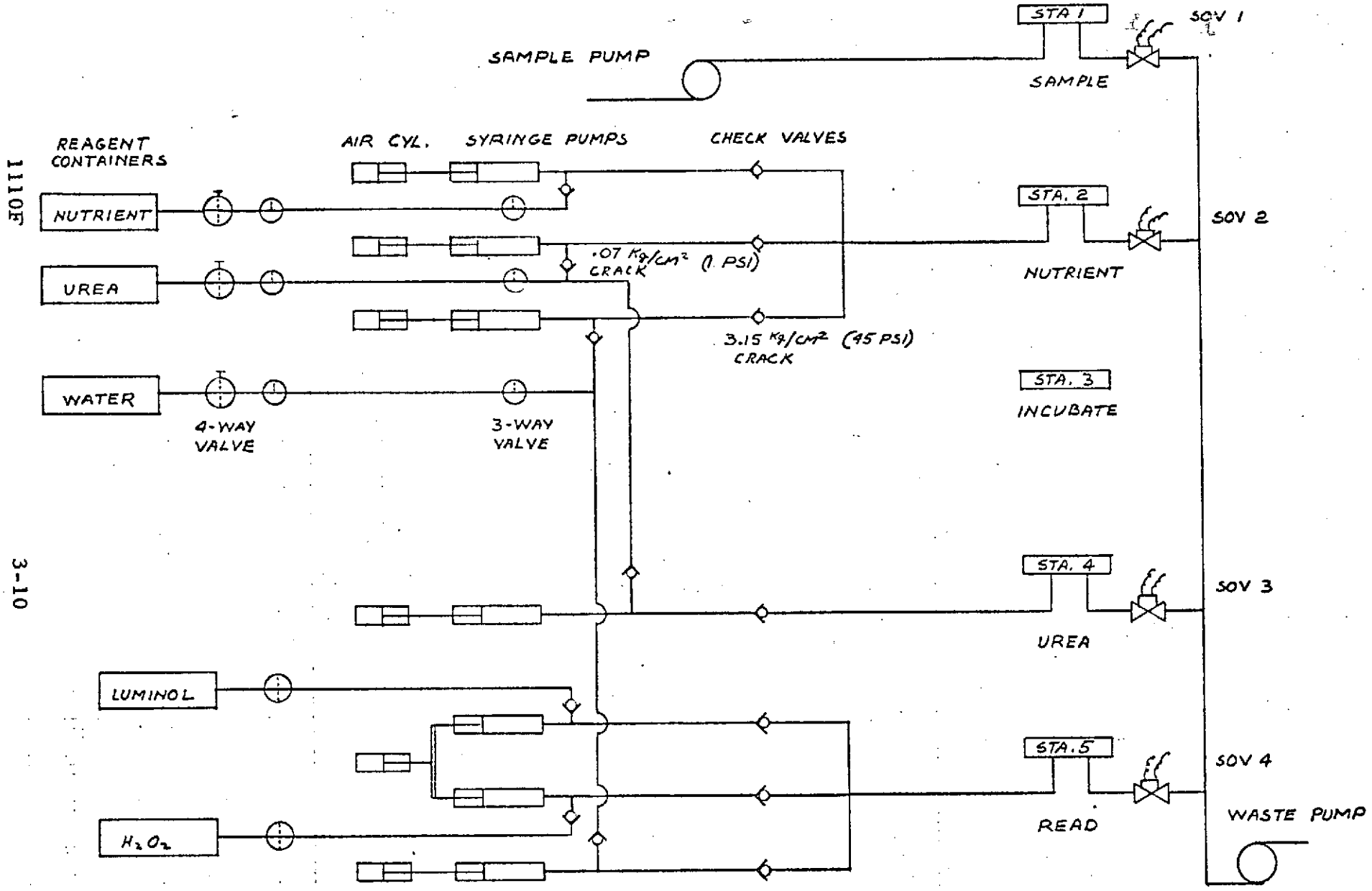


Figure 8 Liquid Flow Schematic

transfer of the capsules from the loading cylinder to the carrier. The capsule loading cylinder has sufficient space for 20 capsules. The capsule is securely held in the carrier by two spring-loaded plungers. The sequence of operations at each station are initiated only when a capsule arrives at that station and trips a limit switch. Any function in that sequence cannot be performed without the previous function being completed. Carriers are located on the chain only where a capsule is required.

(2) Sample Concentration Station

Concentration of the bacteria in a 400-ml water sample is accomplished at this station by pressure filtration through the capsule. A positive displacement pump, used originally to meter the sample fluid, has been replaced by a parastaltic pump. The reciprocating piston pump was found to generate particulates (presumably from the carbon liner) which plugged the filter and led to excessive pressure buildup in the capsule. The difficulty with a parastaltic pump is the variation of flow rate with back pressure up to 30% at 3.5 kg/cm^2 (50 psig) which may introduce an error in the sample volume delivered since the latter is a timed sequence in automatic operation. To avoid this problem the actual volume delivered is recorded and a correction applied to the data.

(3) Nutrient Addition Station

After sample concentration the capsule moves to the next station where a small amount of dextrose broth is passed through the filter. As this capsule passes on to the next station (i.e., the incubator), another (empty) capsule directly behind it moves into position at the nutrient station. A 4 M Urea solution is passed through the syringe needle followed by a sterile water rinse. This post-rinse is intended to sterilize the syringe needle between samples and prevent cross-contamination.

(4) Incubator Station

In the "viable" cycle, the capsule containing the deposited organisms is incubated for two hours at 37°C. The bottom or the capsule is sealed off during the incubation to prevent drying out of the capsule.

Inasmuch as convection could not be used for heating the capsule to incubation temperature in a zero-g environment, this was accomplished by means of a heater rod placed in direct contact with the upper surface of the capsule. Tests described in the Laboratory Support Section indicated that the capsule required approximately three minutes to reach equilibrium temperature and exhibited a temperature variation not exceeding $3/4^{o}C$ over a two-hour heating period. No significant drying out of the capsule occurred during incubation.

(5) Urea Wash Station

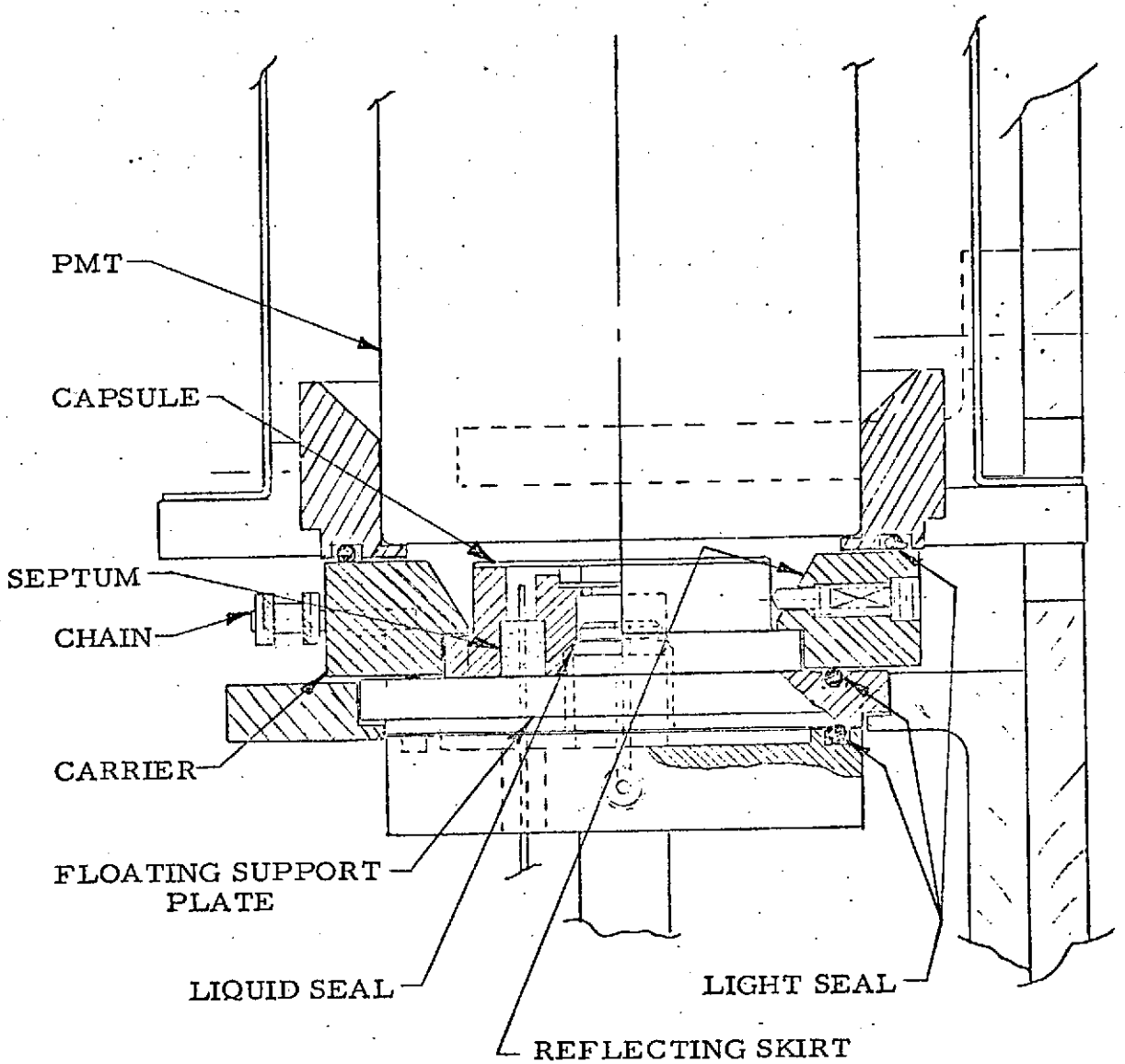
4 M Urea solution is passed through the capsule to wash out the nutrient. Residual traces of the latter could produce a spurious signal since the nutrient broth does contain heme porphyrins.

(6) Reaction/Readout Station

With the photomultiplier tube (PMT) fixed, the carrier containing the capsule moves into position. After the plunger engages the capsule an effective light shield of the capsule and the PMT is achieved by means of three O-rings shown in Figure 9. To insure maximum light-gathering efficiency the capsule is brought close to the surface of the PMT and a reflecting skirt, to capture any light diffracted out the sides of the capsule window, forms an integral part of the capsule carrier.

After the plunger has engaged the bottom of the capsule, voltage is applied to the PMT and a discrete volume of luminol-hydrogen peroxide is injected into the capsule and reacted with the deposited organisms. The generated light is monitored by an open-end photomultiplier tube (EMI 9635B) and the processed signal appears on a strip chart recorder. When operating the instrument in the manual mode, the full analog signal is recorded (i.e., the peak signal and the decayed equilibrium value). In the automatic mode an additional signal appears after about 10 minutes which represents the difference between the maximum peak signal and the decayed equilibrium value. Test results of the previously designed system indicated this method was adequate to resolve the data, however, the presentation of the data for this system could have been better resolved by integrating the analog output over a ten-minute period. Figure 10 is a typical signal of a bacterial challenge.

Figure 9 PMT Station Assembly



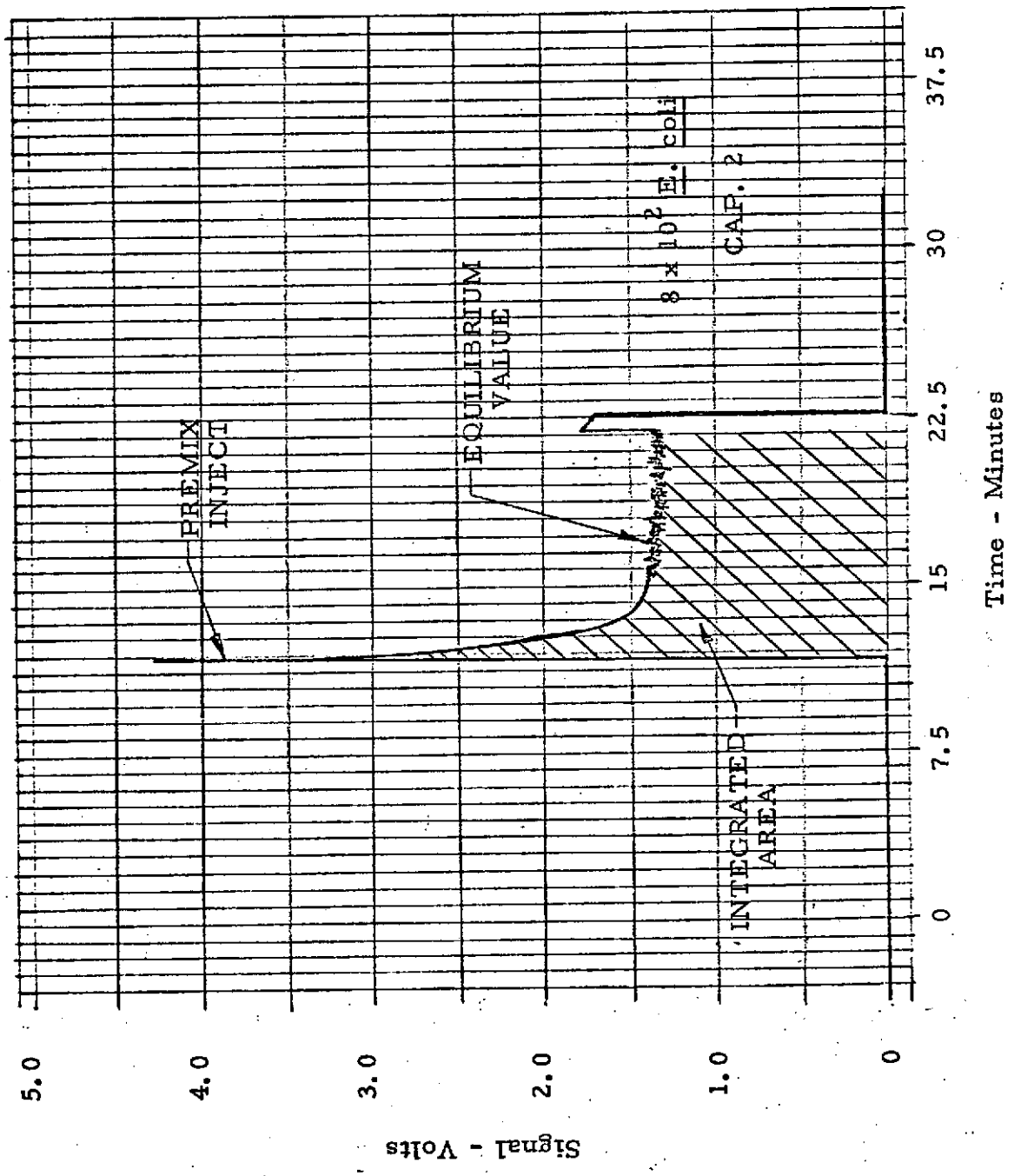


Figure 10 Typical Analog Signal

The initial sharp peak represents a signal of unknown origin and is approximately the same level regardless of the bacterial challenge. The exponential decay after the initial peak represents the challenge level. The slower the decay the higher the bacterial level. Since the initial peaks are approximately the same level regardless of challenge, the peak versus decayed equilibrium technique for resolving the data is of no value. Consequently all the data taken in automatic operation was integrated.

Following reaction with premix reagent power to the PMT is turned off and sterile distilled water then passed through the capsule to remove residual reagent which might react prematurely with the next sample capsule.

(7) Capsule Unload Station

The unload station consists of a small air cylinder to eject the capsule from the carrier and a container with a slit rubber flap at the entrance to store the used capsules.

The times required for the total and viable cycles are summarized in Table 1. It is evident that approximately 1/2 hour is required to process a sample in the "total" cycle and an additional 2-1/2 hours for the "viable" cycle.

The physical dimensions and operating parameters for the integrated bacteria detection system are summarized in Table 2.

b. Reagent Storage and Pump Metering System

The pressurized reagent feed system consists of storage tanks and syringe pumps for injecting the required amount of liquid into the capsule during the processing cycle (Figures 4 through 6).

Retracting and refilling of the syringe pumps are accomplished by maintaining the tank pressure at 1.75 kg/cm^2 (25 psig). Activation of an air-actuated solenoid is used for emptying the syringe contents into the capsule. The cracking pressure of the check valves leading to the capsule are set at 3.15 kg/cm^2 (45 psig). A warning panel light goes on and the pumps fail to retract for filling when the liquid contents of the storage tanks drop below a 10% level.

TABLE 1 LIQUID PROCESSING TIME

Station	Function	Volume (ml)	Time (min) Total	Time (min) Viable
1	Sample Concentration	100	10	10
		400*	20	20
2	Nutrient	5	.25	.25
	Urea Wash	12	.5	.5
	Water Wash	12	.5	.5
3	Incubation	-	0	120
4	Urea Wash	17	.5	.5
5	Premix Reaction	.6	10	10
	Water Wash	10	.5	.5
	**Total 100-ml sample		25	145
	Total 400-ml sample		35	155

*Two lines used on parastaltic pump.

**Some time lost in transporter advance.

TABLE 2 WATER MONITOR SPECIFICATIONS

Width	61 cm (24 inches)
Height	66 cm (26 inches)
Length	102 cm (40 inches)
Weight	102 kg (less than 225 pounds)
Power	115 volt, 60 Hz, 300 watts
Air Supply	7.03 kg/cm^2 (100 psig)
Sample H_2O size	400 ml

Cycle Time:

Unincubated	25 minutes
Incubated	145 minutes

Reagent Storage:

Nutrient	325 ml
Water	2200 ml
Urea	2200 ml
Luminol	65 ml
H_2O_2	65 ml

Reagent Stability:

Nutrient	Hasn't been established.
Urea	At least seven weeks.
Water	At least seven weeks with 30% 4 M Urea.
Luminol	One month.
H_2O_2	One month.

(1) Reagent Containers

Originally the liquid reagents were stored in two different types of containers, namely:

(a) Teflon-bellows container--the luminol and hydrogen peroxide are each stored in a teflon-bellows type container. The bellows with an approximately 75-ml capacity are most conveniently filled by injecting the fluid with a hand syringe (disposable polypropylene plastic syringe) through the rubber septum connected to the 4-way valve of the outer stainless steel container. Liquid is removed through the exit port of the 4-way valve by pressurizing the container on the air side of the teflon bellows. The container cannot be completely emptied in this manner with the residual volume being about 20%. Figure 11 shows a pressure/volume history of the bellows container. For a constant air input pressure the liquid output pressure varies with the volume of liquid expelled. If the last 10 ml of liquid injected into the container is not returned into the syringe the pressure exceeds the downstream check valve cracking pressure and leakage occurs.

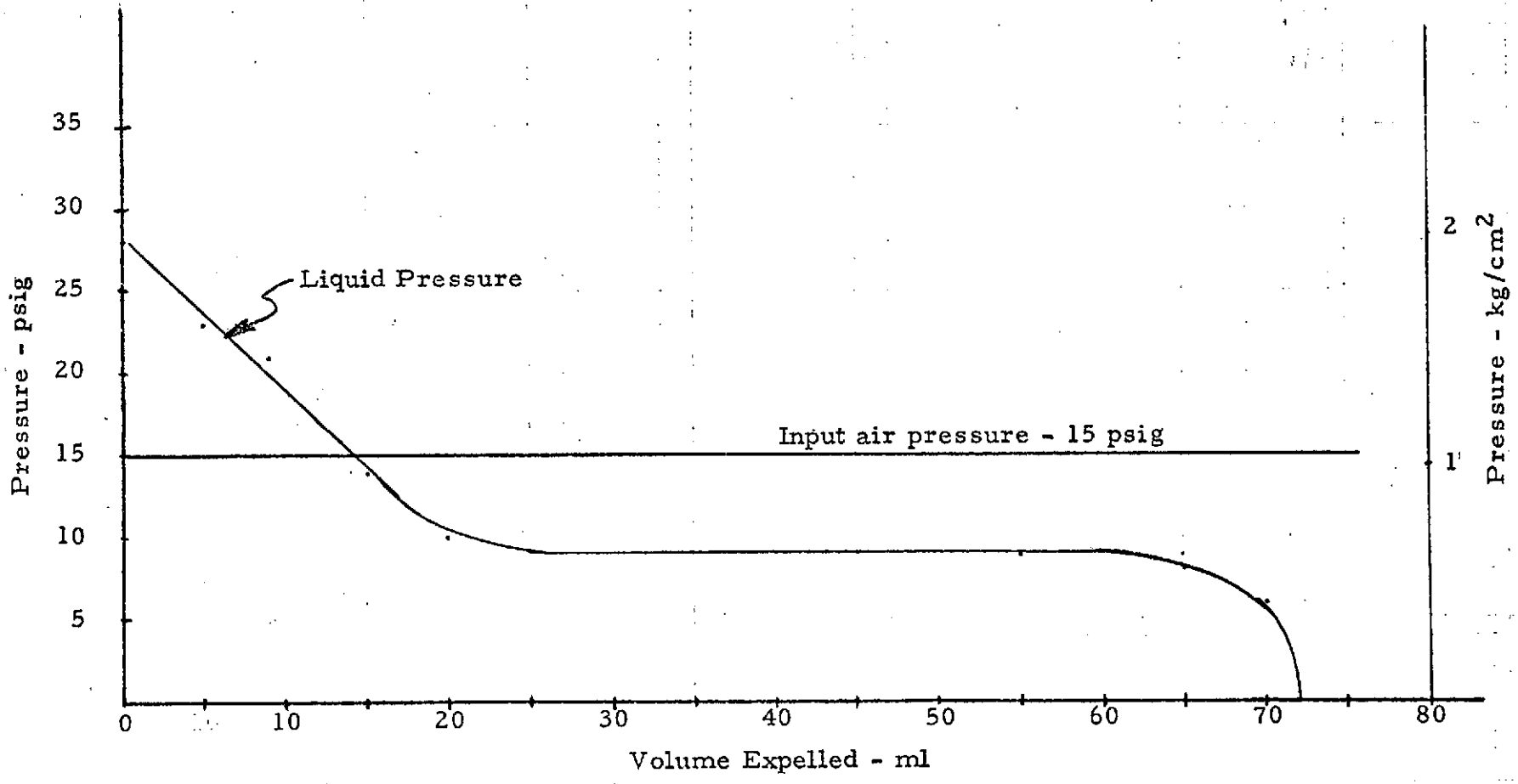
(b) Bellofram containers--the container originally utilized for storing the other liquids (i.e., urea, dextrose broth, and sterile water) was a pressure-activated rolling diaphragm manufactured by Bellofram, Inc. The rolling diaphragm had the advantage of permitting a container design with a minimum holdup volume, low hysteresis and spring rate combined with a high cycle life. Since there is virtually no pressure differential across the Viton bladder the air permeation rate is insignificant.

Filling of these containers is by gravity feed using a sterile plasma flask and transfer set by the procedure outlined in Appendix B. Pressurizing the air side of the bladder is used to eject liquid through the exit port, the residual volume ranging from 5 to 11%.

A number of attempts to sterilize the empty nutrient and water containers initially by autoclaving or use of chemical bactericides proved unsuccessful either because of compatibility problems or thermal instability of the Viton diaphragm to wet heat.

In order to demonstrate the automatic instrument operation, the nutrient container bladder was removed and the container was

Figure 11 Pressure/Volume History Teflon Bellows Container



inverted to allow air pressurization directly on the liquid for bottom expulsion. Filters were installed on the air and liquid side of the container to maintain sterility. Additional work with a new bladder of ethylene-propylene material should be performed to develop the sterile techniques for operation of the nutrient container.

(2) Syringe Pump Metering System

The syringe pump system basically consists of a liquid piston pump extended by a solenoid operated air cylinder with check valves located upstream and downstream to direct the liquid flow from the containers to the capsule penetrating needle. Containers are pressurized to retract the syringe pumps as well as maintain a positive pressure in the liquid system to eliminate outgassing. Upstream check valves crack at 70 gm/cm^2 (1 psig), while the downstream check valves crack at 3.15 kg/cm^2 (45 psig). Operating pressure of the air cylinders for the syringe pumps is 6.3 kg/cm^2 (90 psig).

c. Electrical Control System

The electrical control system is programmed and initiated by the capsule carrier plates by the use of limit switches. As previously described in Section 3.1.2.a. the system has six stations, each with its own sequence of operations as described in Table 3. As a carrier arrives at a station the limit switch is tripped initiating a sequence of events for that station. All functions in that sequence are initiated by the completion of the previous function limit switch action. At the completion of the program for the station, the engaging cylinder is retracted and cannot re-initiate the sequence until the capsule carrier clears the station, thus resetting the program for the arrival of another carrier. Any syringe pumps which have been actuated for the sequence are refilled when the station cylinder is retracted. When all station cylinders are retracted and syringe pumps are filled, the transporter advances one station, thus initiating the next sequence of events. The transporter system will not advance to the next station for the following reasons:

(1) Failure of the syringe pumps to refill properly due to lack of liquid in the containers or piston sticking.

(2) Failure of the station cylinders to retract due to cycle not being completed or sticking of guide pins.

TABLE 3 WATER MONITOR SYSTEM SEQUENCE OF EVENTS

Time (minutes) Total	Time (minutes) Viable	Control Event	Reaction Event
0	0	Start sequence.	Viable capsule advances to Sta 1.
		Cap at Sta 1.	Capsule engaged by Cyl 1 (extended).
		Cyl 1 extended.	Start waste pump, supply pump, open shut-off valve 1, start Timer 1 (20 min).
20	20	Timer 1 complete.	Stop supply pump, start Timer 2 (5 sec).
		Timer 2 complete.	Retract Cyl 1, stop waste pump, close shut-off 1.
		Cyl 1 retracted.	Advance capsule to Sta 2.
		Cap at Sta 2.	Capsule engaged by Cyl 2 (extended).
		Cyl 2 extended.	Start waste pump, nutrient, open shut-off Valve 2.
21	21	Nutrient Cyl extended.	Start Timer 2 (5 sec).
		Timer 2 complete	Retract Cyl 2, stop waste pump, close shut-off 2.
		Cyl 2 retracted.	Advance capsule to Sta 3.
		Waste capsule at Sta 2.	Capsule engaged by Cyl 2 (extended).
		Cyl 2 extended.	Start waste pump, urea, open shut-off Valve 2.
22		Urea Cyl extended.	Start H_2O.
23		H_2O Cyl extended.	Start Timer 2 (5 sec).
		Timer 2 complete.	Retract Cyl 2, stop waste pump, close shut-off 2.

Table 3 (continued)

Time (minutes) Total	Time (minutes) Viable	Control Event	Reaction Event
	22	Viable capsule at Sta 3.	Capsule engaged by Cyl 3 (extended).
		Cyl 3 extended.	Start Timer 3 (2 hours).
	142	Timer 3 complete.	Retract Cyl 3.
		Cyl 3 retracted.	Advance capsule to Sta 4.
		Capsule at Sta 4.	Capsule engaged by Cyl 4 (extended).
		Cyl 4 extended.	Start waste pump, urea, open shut-off Valve 3.
25	144	Urea Cyl extended.	Start Timer 6 (5 sec).
		Timer 6 complete.	Retrace Cyl 4, stop waste pump, close shut-off 3.
		Cyl 4 retracted	
		Capsule at Sta 5.	Capsule engaged by Cyl 5 (extended).
		Cyl 5 extended	Start premix pump, enable PMT, enable Sample A, start Timer 4 (10 min).
35	154	Timer 4 complete.	Enable Sample A-B, cue recorder, start Timer 5 (30 sec).
		Timer 5 complete.	Disable PMT, disable samples and recorder cue, start waste pump, start H_2O, open shut-off Valve 4.
36	155	H_2O Cyl extended.	Start Timer 6 (5 sec).
		Timer 6 complete.	Retract Cyl 5, stop waste pump, close shut-off 4.
36	155	Approximate cycle time.	

(3) Failure to properly load a capsule at the load station, or unload at the unload station.

(4) Failure of the transporter advance cylinder to advance retract completely.

(5) Failure of the limit switches to actuate properly at any of the functions previously noted.

Power failure will cause the system to shut down. The operator must reset the power switch and initiate the proper program after power has been restored. Power failure occurring during the advance cycle will not effect transporter to complete its cycle thus minimizing improper indexing due to loss of power.

The electrical control panel allows for automatic, manual, and station-automatic operation. Automatic operation allows total (hands off) automatic operation. Manual operation is controlled from a manual control box and allows every electrical operation to be initiated independently with lights indicating the function being operated. The manual control box is connected at the control panel to the connector marked "manual output." Lights on the manual control box operate in either the automatic or manual mode of operation. Station-automatic operation allows the switches on the control panel to initiate the automatic operation at any station provided a proper capsule carrier is at the station. Station automatic switches on the control panel and manual switches on the control box are only operational when the auto/man. switch is in the manual mode. Timers are adjustable from the control panel. Green lights indicate which station is in operation. Red lights indicate which syringe pump has not refilled and warns of a possible empty container. All limit switches, solenoids, and lights are powered from 24 VDC.

d. The Electronic Readout System

The readout system consists of a photomultiplier tube (PMT) and electronics to condition the signal for both analog readout and the presentation of signal versus noise. The PMT power source is a high voltage DC power supply set a 1180 volts. The signal electronics allows for attenuation or amplification as required. A peak holding circuit is available for recording the signal versus noise by subtracting base signal remaining after the 10-minute read time from the peak voltage attained during the read cycle. The signal output is available on the front panel and has been attenuated to provide an output of 0 to 5 VDC.

3.2 INSTRUMENT RELIABILITY

3.2.1 Operational and Mechanical Reliability

In evaluating the integrated system reliability the system generally performed quite well, however, several areas of difficulty have occasionally appeared.

a. Capsule failure to load into carrier--due primarily to excess glue remaining after the capsule assembly. This problem has been solved by carefully removing excess glue remaining after capsule assembly at the lid/body glue line. In addition excess material left after the molding operation at the body base may hinder successful loading.

b. Transporter indexing malfunction--occurring primarily when a carrier is leaving the read station. The floating capsule support plate at the read station is provided with clearance under the capsule to allow vertical movement sufficient to provide a light seal at the O-ring. When the needle and plunger is removed after the read cycle, the capsule is occasionally pulled down on the support plate. During transporter advance to the next station the capsule now protruding below the carrier plate may catch on the O-ring and its support ring causing the transport to hesitate. Additional chamfer at the base of the capsule may solve this problem.

c. Station cylinder failure to retract--The guide pins which engage and index the capsule will jam in the capsule holes at the glue-line of the lid/body joint due to excess glue. Capsule guide pin holes should be cleaned out with a #9 drill after capsule assembly.

d. Nutrient sterility--Although not directly associated with the reliable operation of the system it should be noted that the nutrient container sterility problem has not been solved at the writing of this report. The acquisition of an ethylene propylene bladder is in process and should permit the container to be autoclavable, solving this problem.

3.3 UNINCUBATED CYCLE RESULTS

In the course of testing the instrument in automatic operation a large part of the time allotted for testing was spent in attempting to get a system background signal down to a consistent level to adequately determine the

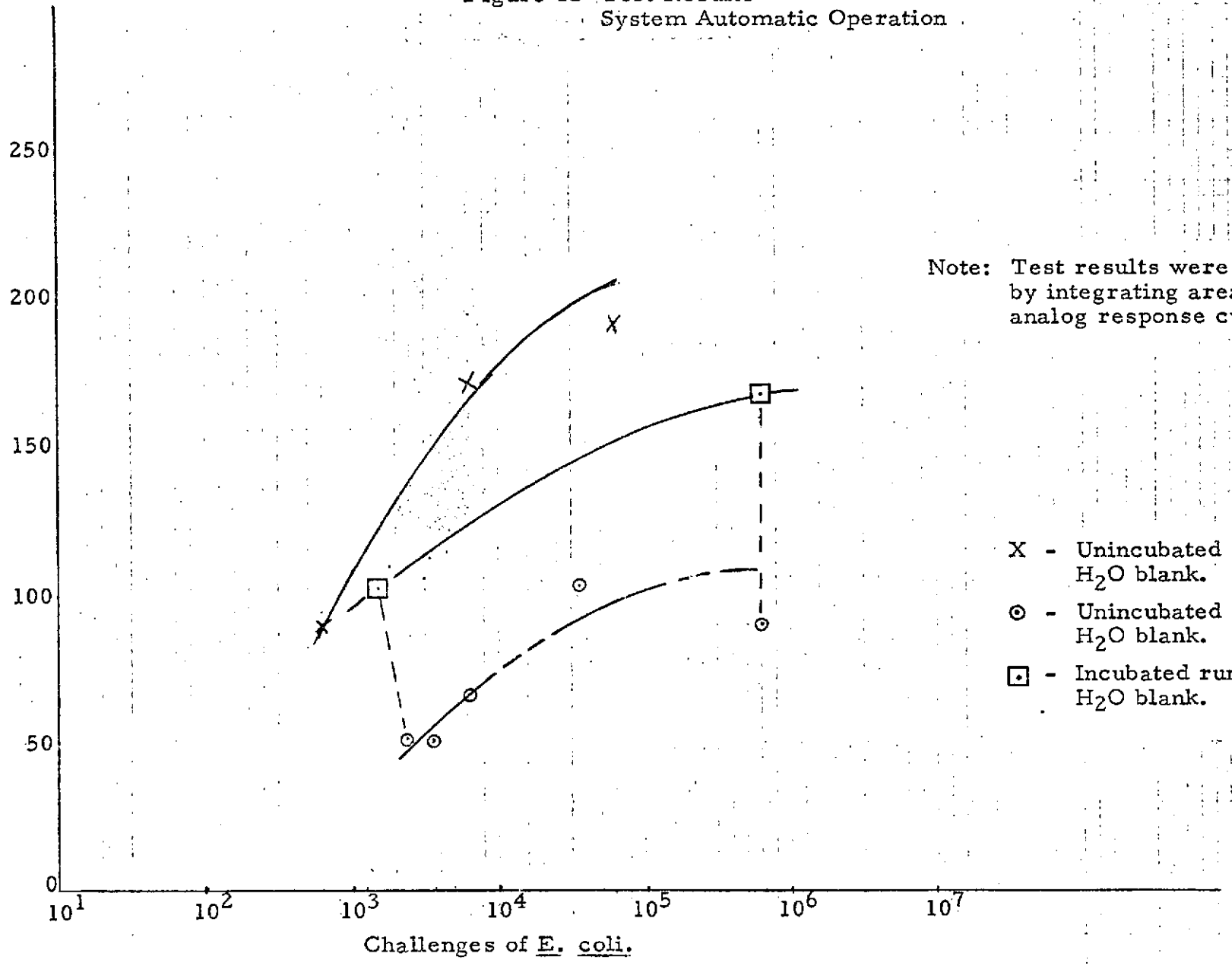
sensitivity of the system. Since the nutrient sterility problem could not be solved because of the EPR bladder lead time, tests were conducted with varying background or contamination levels. Test results in Figure 12 are presented for varying challenges of E. coli and plotted against the percent increase in signal over the water blank. Data was resolved by integrating the area under the trace of the analog signal. Both water blanks and bacterial challenge were run with 100-ml samples using the same water source. For unincubated or total runs two curves are presented. Although the data indicates a substantial loss in sensitivity when high water blanks were recorded, it must be noted that at worst, a 50% increase in signal was recorded at a challenge of 3.8×10^3 E. coli which could easily be resolved by an integrating amplifier recording system. Loss in sensitivity at the higher water blanks could be attributed to the roll-off of the PMT output at high voltage levels (greater than 30 volts) or possibly the piling of glowing bacteria or other background material which may block out some of the available light. With low water blanks, challenges of 8×10^2 E. coli revealed a 90% increase in signal.

Data presented in Figure 13 was taken at a time when water blanks were relatively consistent. A substantial increase in signal is evident when comparing water blanks (100-ml, distilled-deionized water) to bacterial challenges (8×10^2 E. coli). Successful operation of the system by resolving the data in this manner would only be reliable if the water blanks were consistent. Should the system be set up to reference each data point to a preceding water blank, limitations of the system would only be restricted by saturation of PMT.

3.4 INCUBATED CYCLE RESULTS

In order to demonstrate system sensitivity for the incubated or viable cycle, tests were run concurrently with the total cycle runs. Capsules with similar water blank values were run through the system in succession with one used for total cycle and the next subjected to the two-hour incubation. Two viable runs were made under conditions of high water blanks with results revealing a minimum 50% increase in signal over the respective unincubated run at a level 1.9×10^3 E. coli total. A challenge of 8×10^5 E. coli revealed an increase of nearly 80%.

Figure 12 Test Results
System Automatic Operation



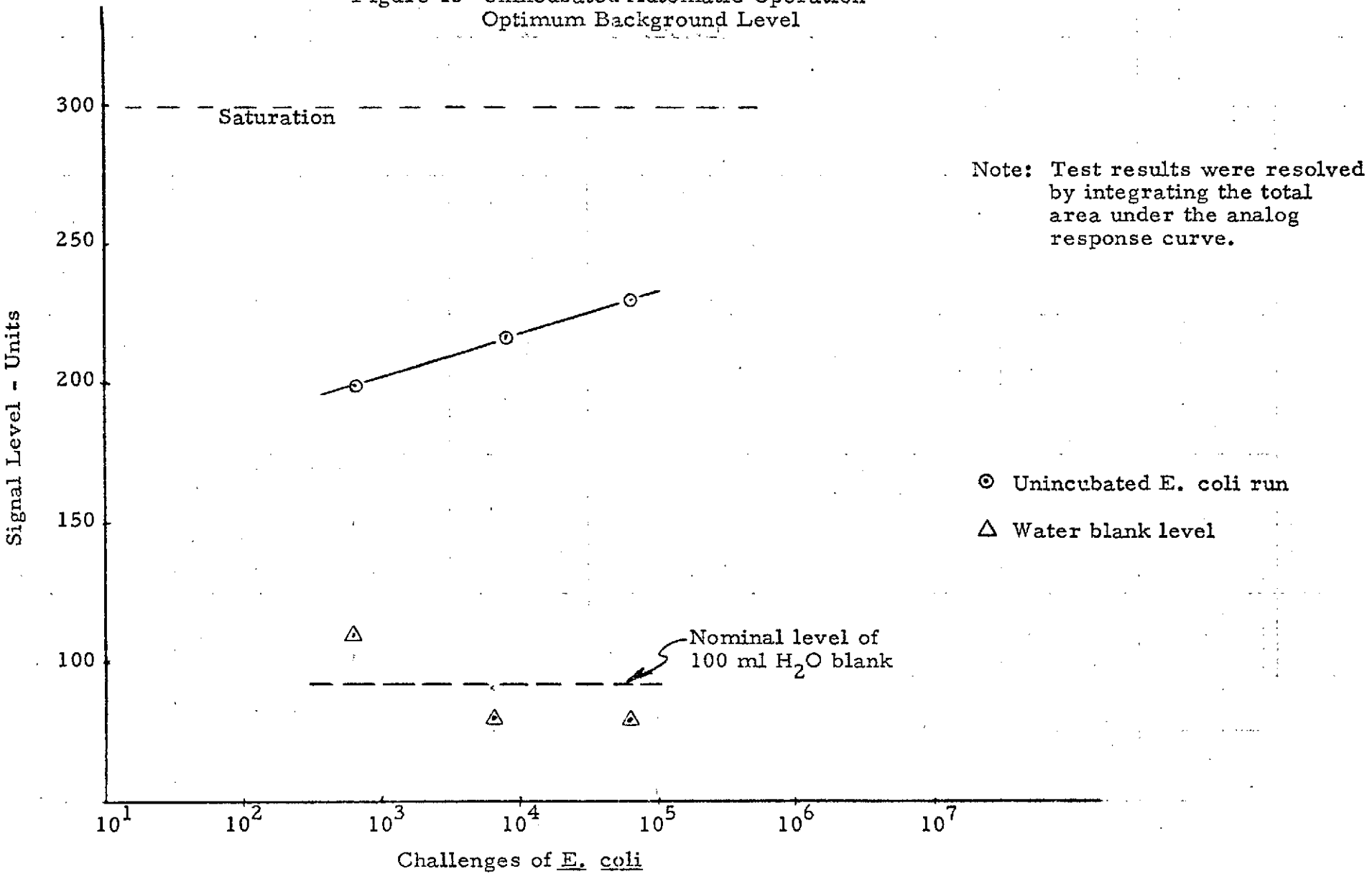
Note: Test results were resolved by integrating area under the analog response curve.

X - Unincubated run with low H_2O blank.

⊙ - Unincubated run with high H_2O blank.

⊡ - Incubated run with high H_2O blank.

Figure 13 Unincubated Automatic Operation
Optimum Background Level



3.5 OPERATION IN SIMULATED ZERO-G ENVIRONMENT

Due to the fact that the nutrient container was not in a zero-g configuration during all-up system test, the simulated zero-g environment could not be demonstrated. During initial start up of the mechanical system (without liquids) tests were conducted at AMB in various positions to assure adequate mechanical operation of the transporter system.

4.0 LABORATORY SUPPORT STUDIES

A number of laboratory studies were carried out in developing the present system design. Included among these were:

Capsule Development

Support Studies for Development of the Processing Station Assembly

Container and Reagent Feed System Development

Reagent Compatibility and Shelf-Life Studies

Specific details follow.

4.1 CAPSULE DEVELOPMENT STUDIES

Specific studies in this area included (a) septum and needle evaluation, (b) pressure profile inside a capsule, (c) obtaining preliminary sensitivity data with a prototype capsule and (d) resolving high background problems associated with the capsule design.

4.1.1 Septum and Needle Evaluation

The feasibility of using Viton rubber as a septum material to minimize the problem of needle plugging was investigated. Silicone "O" ring material, which had been used in the previous program, was found to fragment significantly on being punctured repeatedly with a needle.

For these studies, Viton was selected because it possessed a higher tear and abrasion resistance and contained less filler material than Silicone. It was expected that this combination of properties would make the Viton less susceptible to shearing and fragmentation.

In addition the effect of using a solid-pointed needle with a hole on the side was also evaluated in an effort to reduce obstruction of the needle.

In summary, the results indicated the following:

a. No leakage or needle plugging was evident on flowing 500 ml of liquid through a 20-gauge needle which had previously penetrated the septum five times. This was evident at a variety of flow rates ranging from 1 to 20

ml/min. The Viton also showed less of a tendency to fragment on penetration by the 20-gauge needle.

b. The solid-pointed needle appeared to work satisfactorily with no evidence of plugging. Although this limited test series indicates that both the conventional and solid-pointed needles are equally as good, the latter needle offers a greater margin of safety against plugging.

4.1.2 Pressure Profile Inside a Capsule

The effect of air bubble entrapment on the pressure profile inside a capsule was carried out in support of the capsule design effort. In the procedure used water was pumped (peristaltic) at 22.5 ml/min through a capsule with an Acropor AN 450 membrane filter. After the filter was thoroughly wetted an air bubble was introduced into the line and the pumping continued. A transducer with a pressure pickup (hypodermic needle) mounted inside the capsule cavity was used to monitor the pressure as a function of time. The time required for the pressure inside the capsule to decay to atmospheric after turning off the metering pump was also recorded. These parameters were measured as a function of varying partial vacuums downstream from the filter. Three representative pressure profiles obtained at various downstream vacuums are shown in Figures 14 and 15.

The data (Table 4) indicate that although the ΔP remains relatively constant, the equilibration time for the internal pressure to decay to atmospheric is a function of the vacuum applied, as might have been expected.

4.1.3 Preliminary Sensitivity Data

Preliminary data on the signal response that might be achieved with the proposed septum was obtained with a prototype capsule (Figure 16) and the (syringe) reagent feed shown in Figure 17. Bacterial suspensions and a water control (5 ml of each) were passed through the capsule by hand injection and the capsule then placed in the readout station of the previous program* modified to contain a reflecting skirt. For reaction 0.3 ml each of luminol and hydrogen peroxide were rapidly injected into the capsule and the total light generated on reaction (full area of capsule viewed) monitored by the PMT.

*Contract NAS 9-12548, 27 April 1973.

Figure 14 Capsule Pressure History - No Vacuum

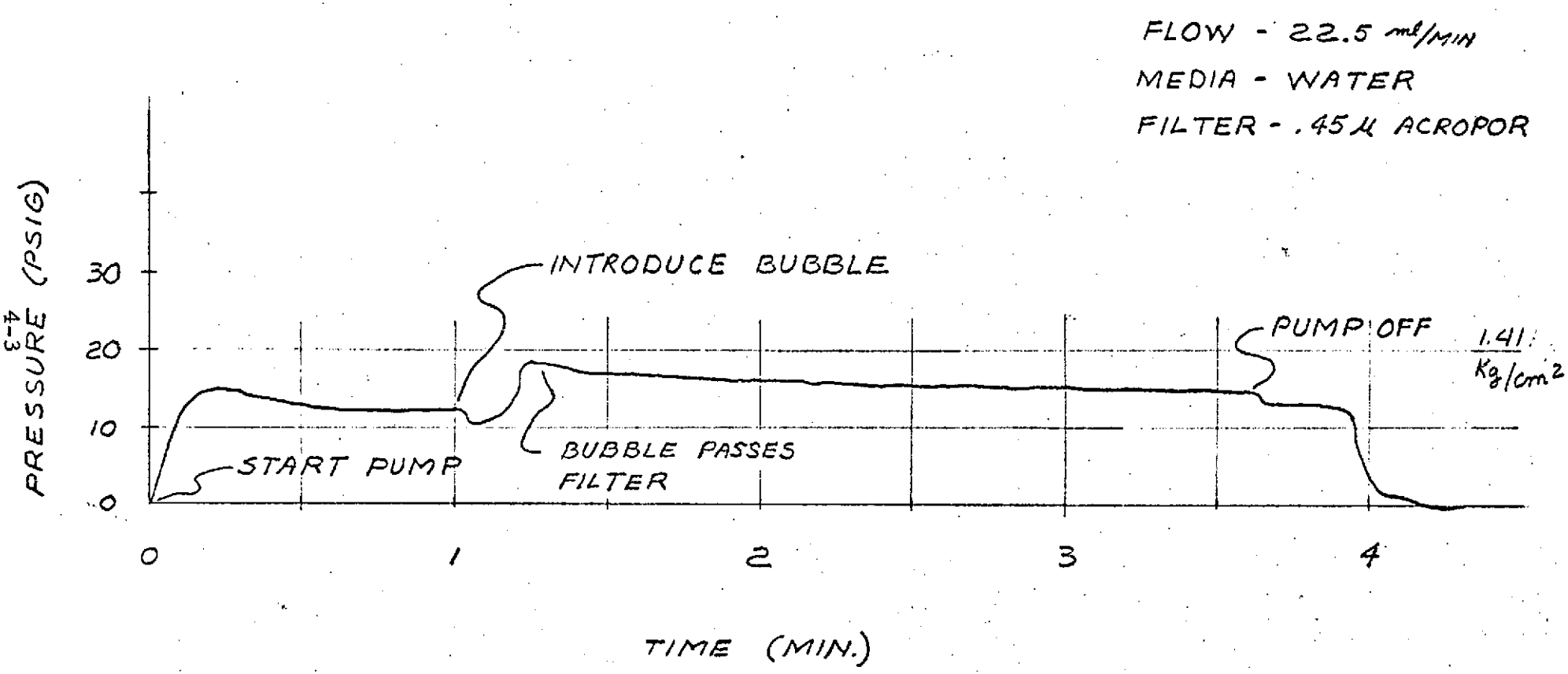


Figure 15 Capsule Pressures - For Various Downstream Vacuums

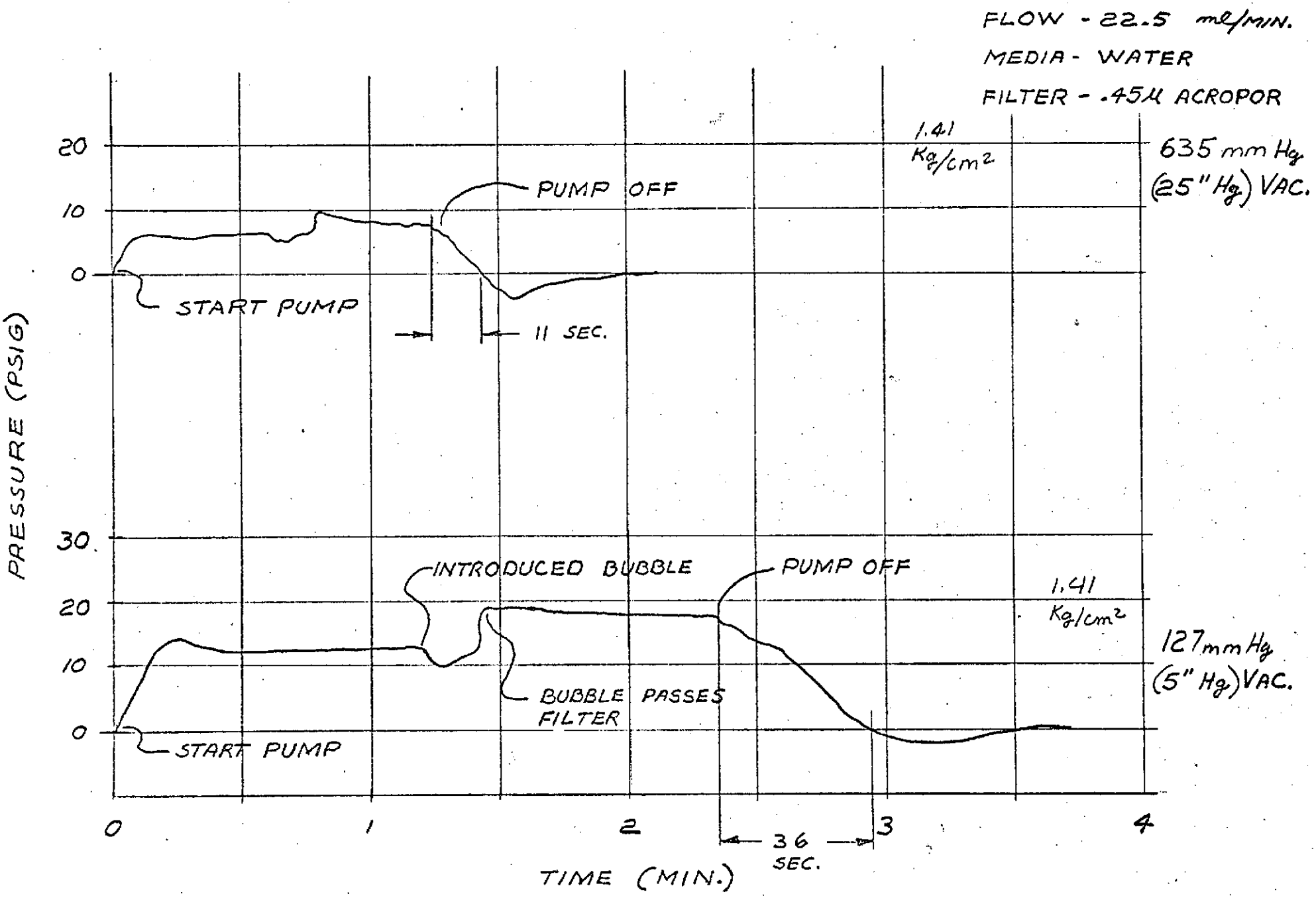


TABLE 4 TABULATION OF CAPSULE PRESSURES FOR VARIOUS VACUUMS

(1) Downstream Pressure kg/cm^2 (psig)	(2) Max. Capsule Pressure kg/cm^2 (psig)	(3) ΔP Across Filter kg/cm^2 (psig)	(4) Equilibration Time (sec)
0	1.36 (19.4)	1.36 (19.4)	45.0
.176 (-2.5)	1.34 (19.2)	1.52 (21.7)	38.5
.344 (-4.9)	1.30 (18.5)	1.64 (23.4)	34.5
.520 (-7.4)	.95 (13.5)	1.47 (20.9)	27.0
.692 (-9.9)	.81 (11.5)	1.50 (21.4)	13.5
.852 (12.2)	.70 (10.0)	1.56 (22.2)	11.0

(1) Denotes vacuum downstream of filter.

(2) Denotes upstream pressure in the capsule cavity.

(3) Summation of columns (1) and (2).

(4) Indicates time to reduce internal pressure to atmospheric.

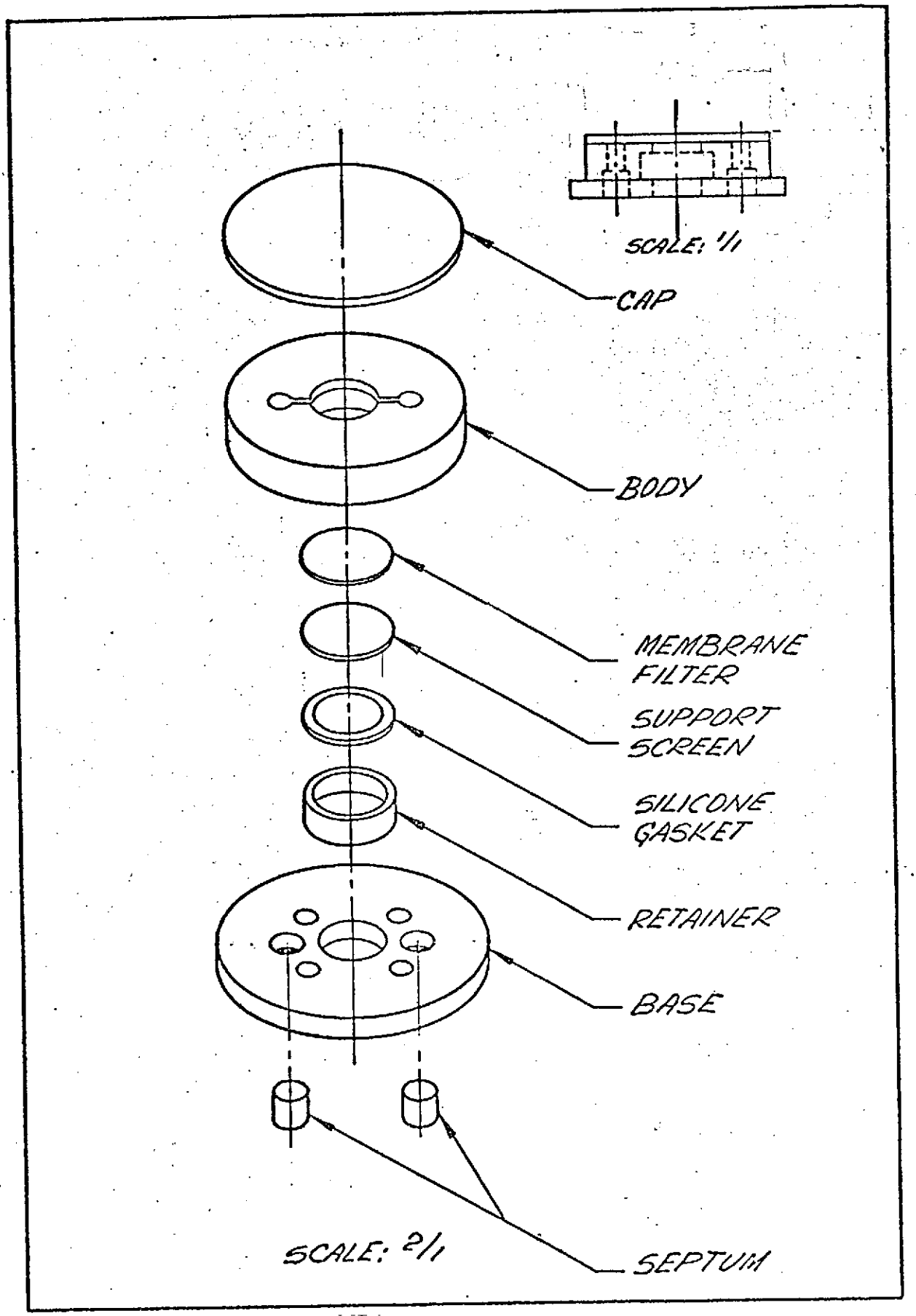


Figure 16 Capsule (Prototype)

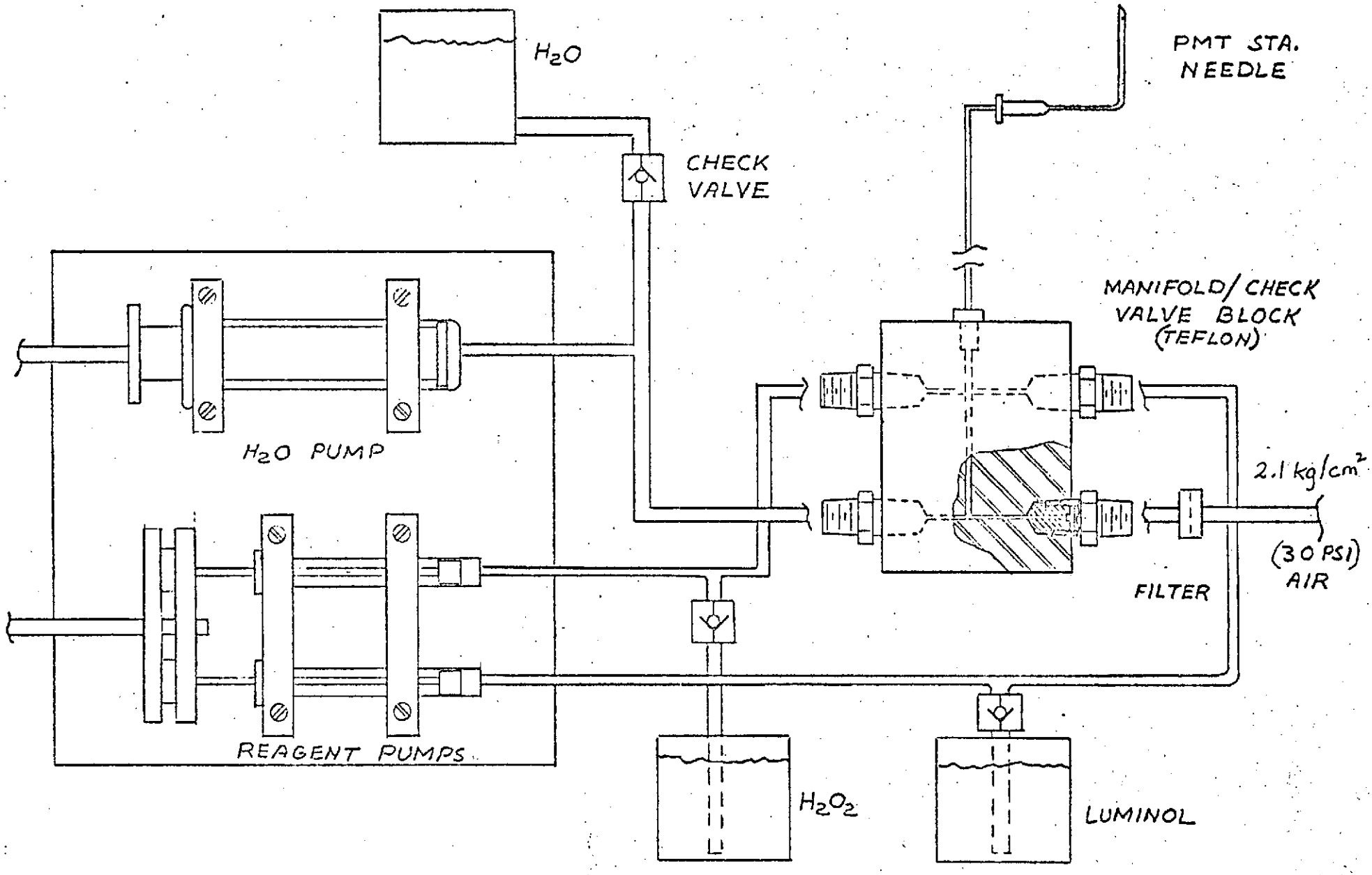


Figure 17 Reagent Feed System (Laboratory Prototype)

For the series shown in Table 5 and 6 air was not used to clear the capsule or manifold line (between mixing block and needle) of resicual liquid prior to reaction. However, the total volume of reagent injected (0.6 ml) was sufficient to displace this residual holdup volume of 0.4 ml.

The results shown for the initial series in Table 5 indicate that total bacterial challenges of the order of 10^4 cells can be readily detected over a water control. A second series (Table 6), run under more controlled conditions, indicated that a total bacterial challenge of 6×10^3 E. coli produced an overall average net signal of 11.5 volts. Assuming 6×10^3 cells contained in a 400-ml sample volume, then one should be able to detect 5 cells/ml with a 95% probability.

Studies in the previous program* suggested that improved sensitivities might be achieved by removing residual liquid from the manifold line and capsule prior to reaction with premix. Apparently entrapped liquid in the capsule could lead to channelling of the premix reagent and incomplete reaction. Removal of this residual liquid might be accomplished by incorporating a step in the processing sequence in which filtered air automatically purges the residual urea from the capsule after the urea wash at the fourth station.

It was observed, however, that with the present system, if the residual liquid is removed from the manifold line and capsule prior to reaction with Premix, the small liquid volume (0.6 ml) injected under the present arrangement is insufficient to reach the capsule because of an air lock. A larger volume of liquid (~2-3 ml) is required to build up sufficient pressure to force the bubble through the filter surface. Actual pressure measurements taken inside the capsule cavity indicate that 1.96 kg/cm^2 (28 psig) is required to force an air bubble through a filter wetted with water. Experiments with a capsule design in which a swirling motion was imparted to the incoming liquid was achieved by having the inlet channel tangential to the perimeter of the reaction chamber. This tended to rid the capsule of air bubbles more effectively. The swirling action tended to dislodge smaller bubbles entrapped on the walls of the chamber (without necessarily touching the filter surface) and causing them to coalesce into a single large bubble. The latter would have a

*AMB Final Report, "Tape Cassette Bacteria Detection System," Contract NAS 9-12548, 27 April 1973, pp. 34 and 35.

TABLE 5 SIGNAL RESPONSE TOWARD E. coli

Total Bacterial Challenge, E. coli	Net Signal, Volts
6×10^5 Cells	50 (Saturation)
1.2×10^5 Cells	40
1.2×10^5 Cells	32
6×10^4 Cells	19
1.2×10^4 Cells	16
6×10^3 Cells	2.5

NOTE: 0-50 Volt span

TABLE 6 SIGNAL RESPONSE TOWARD E. coli

Total Bacterial Challenge, E. coli	Net Signal, Volts	
1.2×10^4 Cells	15	
6×10^3 Cells	12	Av = 11.5 (S^{**} = 2)
6×10^3 Cells	14	
6×10^3 Cells	9.5	
6×10^3 Cells	9.5	
6×10^3 Cells	12.5	

NOTE: 0-50 Volt span

**S = Standard Deviation

greater probability of contacting the filter surface and being forced through when the head pressure reached the bubble point. A swirling motion also assured that all of the filter surface would be contacted by reagent. This feature was incorporated in the new filter design.

4.1.4 High Background Signals with Production Capsules

A preliminary evaluation of assembled and pre-cleaned molded capsules was made by reacting them with luminol-hydrogen peroxide reagent at the readout station. Excessively high background signals (30 volts) were obtained and these were traced to a number of causes described briefly below.

a. A large contribution to the background signal was found to be caused by Premix reagent contacting the underside of the capsule (i. e., the unclean areas between the capsule filter support and the O-ring on the plunger). Chemiluminescence generated in this area was being reflected up through the body of the capsule. Two approaches used to reduce the background signal from this source were the use of (a) a black pigmented capsule body (clear lid) and (b) the use of a light shield in front of the PMT with a 10 mm hole in the center. A preliminary evaluation of both of these approaches using a procedure in which the luminol-hydrogen peroxide reagent was hand-injected into the capsule at the readout station indicated (see Table 7) that the use of the black capsule body is more consistent in producing lower reagent blanks with little, if any, significant loss in sensitivity (i. e., net signals of 7 to 12 volts were obtained with a fixed bacterial challenge of E. coli). The use of a black molded capsule body helped reduce the background signal caused by Premix contacting any portion of the contaminated capsule bottom.

b. The silicone gaskets as supplied by Millipore were found to be undersized permitting dead-end channelling of fluids in the reactor cavity and wicking of fluids under the gasket. This prevented complete cleansing of the reactor cavity by luminol-hydrogen peroxide pre-treatment process. The problem was solved by machining the top surface of the capsule body to accommodate the new gasket dimension specifications for proper gasket sealing action.

c. A contaminated mixing block at the readout station also contributed to the high background signals. Cleansing of the teflon mixing block

TABLE 7 SIGNALS OBTAINED WITH CLEAR AND BLACK BODIED CAPSULES (HAND INJECT PROCEDURE)

		Reagent Blank,* volts		Net Signal** (E. coli), volts	
		with PMT Shield	without PMT Shield	with PMT Shield	without PMT Shield
A.	Clear Capsule				
	(1) Without Screen	7-10	20-35	12	not determined
	(2) With Screen	14-17	28	10	not determined
B.	Black-Bodied Capsule				
	(1) Without Screen	4-7	14	10	7-10
	(2) With Screen	9-10	9-12	9	7-10

*Reagent Blank--signal obtained 7 seconds after introducing luminol-hydrogen peroxide into a clean capsule.

**Net Signal obtained on subtracting signal for a fixed bacterial challenge contained in 5 ml filtered, distilled water, from a water blank of 5 ml (nutrient and urea washes after depositing organisms on filter omitted).

assembly (which contains channels of .5 mm (0.020") diameter) has led to reagent blanks with system Premix of 15 volts (black molded capsule body, without stainless steel screen or PMT shield) compared to 14 volts under comparable conditions by the hand-inject procedure.

d. Chemiluminescence resulting from contact of Premix with uncured adhesive (PS-30, IPS) used in cementing the lid to the capsule body has contributed to the background signal. Capsules are now post-cured overnight at 37°C prior to use to minimize this problem. While adhesives used on the previous program (i.e., #2395 and #1807--solvent types from IPS) show lower reactivity toward the luminol-hydrogen peroxide reagents, it is more difficult to achieve a satisfactory bond with these adhesives in the present capsule design. The PS-30 currently being used is a two-component, polymerizable acrylic (acrylic monomer plus peroxide catalyst) which is more viscous than the other glues and although possessing superior sealing characteristics, causes frequent plugging of the inlet channels in the capsules. The channel was opened up to solve this problem.

4.2 SUPPORT STUDIES FOR DEVELOPMENT OF PROCESSING STATION ASSEMBLY

4.2.1 Sample Feed Pump

A pump manufactured by Fluid Metering, Inc., was originally intended for metering the 400-ml water sample into the capsule. This pump is basically a reciprocating piston pump in which the piston reciprocates and rotates synchronously, completing one pressure and one suction stroke with each revolution. The pumping accuracy is based on a positive displacement mechanism without the use of valves. Pumping variations of less than 1% are claimed for most liquids. To minimize flow variations a ceramic piston specially matched to the 316 SS cylinder (with carbon liner), was utilized.

A number of studies were conducted in which a pressure gauge was inserted between this sample pump and a membrane filter (Acropor AN 450, 13 mm diameter, 0.45 μ pore size) in a Swinny holder (to simulate a filter capsule). Excessive pressure was built up (i.e., 4.2 kg/cm^2 (60 psig) or greater) in less than the 20 minutes normally required to filter a 400-ml

volume through the filter capsule. Microscopic examination of the filter indicated buildup of a gray deposit, presumably of carbon particulates from the carbon lines of the pump cylinder. Dismantling and cleaning of the pump components followed by repeated use failed to eliminate the excessive pressure buildup. The Fluid Metering Pump was replaced by a Buchler Parastaltic Pump. An alternate replacement which might be considered is a Gorman Rupp positive displacement bellows pump (Model M 13300-5).

4.2.2 Incubator

To evaluate the performance of the incubator station a test capsule, containing an embedded copper-constantan thermocouple was filled with dextrose broth. The system temperature control was adjusted to maintain a temperature of 37°C in the capsule filter area. The instrument capsule was then indexed to the incubator station and the following data recorded.

1. Rise time from ambient to 35.5°C--3 minutes.
2. Stabilized at 36.5°C--10 minutes.
3. Maintained at 36.5°C $\pm$ 0.75°C--2 hours.
4. No significant drying out of capsule (80% of void filling retained) occurred during the 2-hour incubation.

4.2.3 Photomultiplier Tube Assembly

During the processing of the viable cycle, the photomultiplier tube would normally be exposed to shielded ambient light for periods up to three hours. The effect on sensitivity of exposing the PMT to ambient light was evaluated to determine whether a shutter would be required.

In the procedure used, a fixed light (incandescent lamp behind a blue filter with peak transmission at 430 μ, the wavelength for luminol chemiluminescence) was used to produce a given signal from the PMT (which had been equilibrated in the dark for at least 24 hours). The signal output was checked again after exposing the PMT to ambient light for 30 minutes and 3 hours (the maximum cycle time between samples) respectively. The results, shown in Table 8, indicate less than a 7% variation between the various signals, indicating that a shutter for the PMT would not be required.

Table 8

EFFECT OF EXPOSING PMT TO AMBIENT LIGHT

PMT Treatment	Peak Voltage
Original Unexposed PMT Output (Control)	4.7 volts
30-Minute Exposure to Ambient Light	4.8 volts
3-Hour Exposure to Ambient Light	4.4 volts

4.3 REAGENT SUPPLY SYSTEM STUDIES

4.3.1 Pressurized versus Non-Pressurized System

The partial pressures required to cause outgassing of the liquid reagents were determined in order to establish what the minimum absolute pumping pressure would have to be in order to prevent bubble formation in these liquids. The results, shown in Table 9, indicate that the two liquids which outgas most readily are the luminol and dextrose solutions, i.e., 571 mm Hg and 635 mm Hg (22.5" and 25" Hg Abs, respectively). Since the cracking pressures of the check valves in the reagent supply is ~711 mm Hg (~28" Hg), operating an unpressurized reagent feed supply would be marginal with respect to outgassing of these two liquid reagents. Consequently, use of a pressurized reagent feed system was considered advisable.

4.3.2 Container Sterilization Studies

Prior to the initial filling of the system containers an attempt was made to sterilize the water and nutrient containers by autoclaving with steam at 1.5 kg/cm^2 (15 psig) at 394°K (250°F) for 15 minutes. Failure of the diaphragms was noted (i.e., separation of the Viton film from the polyester backing) to occur at points where the Viton film was under compression. A check with the supplier (Bellofram, Inc.) revealed that the diaphragm could take wet heat up to 349°K (170°F), or dry heat up to 561°K (550°F); it would not withstand the steam generated during autoclaving. In the presence of steam a magnesium oxide filler in the Viton picks up moisture and expands breaking the bond to the polyester backing.

An alternate approach for sterilizing the reagent container assembly would be by use of dry heat at 433°K (320°F) for two hours. The difficulties associated with this technique involves the ability to transfer the nutrient to the container while maintaining sterility and the ability of the stainless steel valves to maintain integrity after repeated temperature cycling to 433°K (320°F) for sterilization.

The technique which shows the best promise would be to use an EPR bladder and autoclave at 394°K (250°F) and 1.05 kg/cm^2 (15 psig) for 15 minutes with the container partially filled and a .45-micron filter on the

TABLE 9 ABSOLUTE PRESSURES AT WHICH LIQUID REAGENTS OUTGAS *

4 M Urea	12.6 cms Hg Abs (5" Hg Abs)
Dextrose Broth	63.0 cms Hg Abs (25" Hg Abs)
Luminol Solution**	57.2 cms Hg Abs (22.5" Hg Abs)
Hydrogen Peroxide Solution≠	32.8 cms Hg Abs (13" Hg Abs)

*Ambient temperature, 23°C

**0.16 mg/ml Luminol

≠0.36%

liquid side. A valve would be mounted downstream of the filter to allow flushing of the nutrient lines with urea for downtimes.

Chemical sterilization provides another alternative; however, the requirements for an acceptable procedure is one which is chemically compatible with the materials of construction and one which does not leave a residual which would interfere with the chemiluminescence reaction. Also it should be non-toxic to humans, to prevent contaminating the water supply if it is recycled.

A number of chemical bactericides which were considered are shown in Table 10.

Of all the chemical agents shown in Table 10, ethyl alcohol and formaldehyde appear promising compromises. Apart from their effectiveness as bactericides in the present system, the maximum concentration which could be tolerated without producing interference with the chemiluminescence would have to be ascertained.

In an initial evaluation using ethyl alcohol the nutrient and water container assemblies were pre-soaked in (anhydrous) ethyl alcohol, the latter pumped out and then refilled with sterile nutrient and water, respectively. Samples withdrawn after one week at ambient temperature indicated the presence of fungal growth (possibly yeast) in the nutrient container and bacterial growth in the water container.

A second study performed using 70% ethyl alcohol as the germicidal agent (followed by three sterile distilled water rinses prior to addition of sterile nutrient) indicated that although a contamination-free transfer was accomplished bacterial growth became evident in the nutrient after only 24 hours at ambient temperature.

While greater success might be achieved with the other bactericides (i.e., formaldehyde in ethyl alcohol) it would be more expedient to modify the pumping system to permit use of non-pressurized water and nutrient reagents which are sterilized daily. The other reagents which are not prone to bacterial growth (i.e., the urea, luminol, and hydrogen peroxide) could be dispensed using the present pressurized arrangement. Any outgassing of the

Table 10

CHEMICAL ANTI-MICROBIAL AGENTS

Agent	Comments
Ethylene Oxide	Reacts with Viton diaphragm.
Phenols or Phenolic Compounds	Interferes with chemiluminescence in ppm range; inert against some bacterial spores.
Halogens (iodine, chlorine, or hypochlorites)	Interferes with chemiluminescence in ppm range; inert against some bacterial spores.
Heavy Metals	
a. Mercuric or Mercurous Ion	Toxic to humans.
b. Silver Ion	Bactericidal at dilutions of 1:1000. Interferes with chemiluminescence in the ppm range; inert against some bacterial spores.
Quaternary Ammonium Compounds	Compatibility problem with Viton; inert against some bacterial spores.
Alcohols (Ethyl Alcohol)	50 to 70% most effective; inert against some bacterial spores.
Formaldehyde	8% solution in water or 70% ethyl alcohol; vapor polymerizes at room temperature forming a solid. Effect on chemiluminescence not known.
4 M Urea	More effective as a bacteriostat (i.e., inhibits bacterial growth) than as a bactericide. No significant effect on chemiluminescence.
Sodium Azide	Concentrations of 0.02% inhibit growth of Gram-negative bacteria but not Gram-positive organisms.

water or nutrient which might result as a consequence of using a non-pressurized system would not be critical for these reagents.

An alternate approach utilized for the nutrient container was to remove the diaphragm, fill with nutrient and then sterilize the whole assembly by wet autoclaving at 377°K (220°F) for 15 minutes. Pressurized sterile air was then used to expel the fluid from the container which had been inverted.

4.3.3 Reagent Shelf Life Studies

The shelf life and compatibility of the liquid reagents with various materials of construction were investigated. Luminol, hydrogen peroxide, urea, and nutrient solutions, at the concentrations at which they are intended to be used, were placed in contact with pre-cleaned strips of 316 stainless steel, Viton B, EPR (ethylene propylene rubber) and PVC (Polyvinyl chloride-plasticized). Teflon and Kel F, used in the reagent containers for luminol and hydrogen peroxide, were not examined since these are known to be inert toward the respective reagents. Materials currently being employed in the system reagent containers are as follows:

Luminol	Teflon, Kel F
Hydrogen Peroxide	Teflon, Kel F
Dextrose Broth	SS (316), Viton B
Urea	SS (316), Viton B

EPR is being considered as a backup for Viton B. PVC (plasticized, Cutter Labs) was also investigated as it was originally intended for use in a reagent bag for refilling the system containers. Samples were prepared and stored as follows:

Luminol, H_2O_2, and Urea were stored in sterile polypropylene (PP) containers with snap-on lids. The air space above the liquids was displaced with dry nitrogen gas prior to capping. This PP container was then placed in an outer envelope of unplasticized PVC which was subsequently heat-sealed. The latter is a more effective barrier for permeation by CO_2 and O_2 which are known to be deleterious to the respective reagents. The sealed samples were then stored at ambient in the dark.

Nutrient was then placed in contact with these materials in glass containers with polyfoam stoppers and then heat-sterilized at 377°K (250°F) for 15 minutes. The sterile samples were stored at ambient in the dark.

The tests run and results obtained on samples stored eight weeks at ambient temperature are described below.

1. Luminol and Hydrogen Peroxide

Degradation of these reagents was evaluated by reacting the aged luminol and H_2O_2 against a single bacterial level of E. coli in an AMB Chemiluminescence Detector. In the latter, 0.2 ml each of luminol and H_2O_2 are rapidly injected with a spring-loaded syringe into an aqueous suspension of E. coli contained in a small test tube mounted in front of a photomultiplier tube. The PMT output is fed into an oscilloscope where the maximum voltage is recorded. One day old luminol and freshly prepared H_2O_2 were used as controls. Since aging of one component of the luminol-H_2O_2 mixture is sometimes not discernable when both the aged luminol and H_2O_2 are used together in a test, aged luminol is also checked against unaged H_2O_2 and aged H_2O_2 against unaged luminol. The results, summarized in Table 11, can be interpreted as follows:

a. The approximately 55% increase in signal observed for aged versus unaged luminol and hydrogen peroxide can be attributed to formation of a luminol-peroxide intermediate on aging as a result of air oxidation of the luminol which increases sensitivity. Characteristically, on aging, luminol sensitivity will increase gradually maximizing several weeks after mixing, followed by a gradual decline.

b. Hydrogen peroxide does show considerable degradation after eight weeks at ambient without any material in contact with it other than the polypropylene container probably as a result of the higher dilution currently being employed. Under the previous program, the H_2O_2 was stored as a 1% solution and then diluted with H_2O prior to reaction; in the present program the H_2O_2 is stored at approximately a 0.35% solution. Luminol is also being used in a more diluted form (0.16 mg/ml versus 1 mg/ml). A more careful dilution to exclude trace organics and metal and/or the use of a preservative (ethylenediaminetetracetate) may inhibit this degradation.

Table 11

EFFECT OF AGING LUMINOL AND H_2O_2 SOLUTIONS ON CHEMILUMINESCENCE SENSITIVITY*

Mixture	Overall Chemiluminescence Signal, Volts**
Unaged luminol + unaged H_2O_2 (control)	13.6
Aged luminol + aged H_2O_2	22.5
Aged luminol + unaged H_2O_2	20.7
Aged luminol + aged H_2O_2 + Viton B	14.5
Aged luminol + unaged H_2O_2 + Viton B	21.5
Aged luminol + aged H_2O_2 + SS	3.8
Aged luminol + unaged H_2O_2 + SS	21.0
Aged luminol + aged H_2O_2 + EPR	11.0
Aged luminol + unaged H_2O_2 + EPR	11.0
Aged luminol + aged H_2O_2 + PVC	12.0
Aged luminol + unaged H_2O_2 + PVC	10.5

*All aged samples stored at ambient temperature for 8 weeks.

**Average signal obtained on reaction with a single concentration of E. coli in AMB Static Chemiluminescence device; not corrected for water blank.

In any event the decrease in the effective peroxide concentration appears to be more than offset by formation of the luminol-peroxide intermediate so that if the aged materials are used together the net result is still an increase in sensitivity over the unaged mixture of H_2O_2 peroxide and luminol.

Referring to the data in Table 11, the effect of the other materials in contact with these agents is as follows:

On Luminol:

Polypropylene, Viton B, Stainless Steel (316), EPR, PVC (plasticized)

(least degradation ——————————→ greatest degradation)

On H_2O_2:

Cannot be assessed in this series because of the extensive degradation exhibited by aged control (in polypropylene).

On Luminol and H_2O_2 (if aged luminol used with aged H_2O_2):

Polypropylene, Viton B, PVC, EPR, SS (316)

(least degradation ——→greatest degradation)

2. 4 M Urea

Since the urea is used as a wash to remove excess nutrient in the present protocol, the most effective test of its efficacy would be an end-to-end run in the system itself comparing the signal obtained with aged urea in a water blank with the signal obtained using fresh 4 M Urea. Incomplete removal of nutrient would be indicated by an excessively high signal in the water blank. An alternative test, albeit less desirable, which was utilized was to compare the signals of aged and unaged urea containing the same E. coli concentration. These conditions would approximate those which occur when the sample capsule containing residual 4 M Urea from the urea wash reacts with luminol-H_2O_2 at the readout station. The results using this test have been summarized in Table 12.

The data indicate that after eight weeks at ambient some alteration in the urea can be expected in contact with stainless steel and Viton B

Table 12

EFFECT OF AGING 4 M UREA AT AMBIENT ON THE CHEMILUMINESCENCE SIGNAL*

Sample	Average Chemiluminescence Signal, Volts
Unaged 4 M Urea	22
Aged in Polypropylene (PP) (Control)	18
Aged in PP + SS (316)	17**
Aged in PP + Viton B	12
Aged in PP + PVC	8
Aged in PP + EPR	3**

*All aged samples stored at ambient temperature for 8 weeks. All samples contained equal concentration of E. coli and checked in AMB Chemiluminescence device using fresh luminol and hydrogen peroxide.

**Aged Urea solutions slightly opalescent; all others clear.

(present materials currently being used in storage container). Since the containers are replenished every 10 days, it is questionable whether the effect would be significant.

3. Nutrient (Dextrose Broth)

Degradation of the dextrose broth on storage would be reflected by a decrease in its ability to support bacterial growth. Samples of sterile broth, aged for eight weeks at ambient temperature in contact with each of the various materials, were inoculated with discrete challenges of E. coli--actual bacterial concentration in each sample determined by direct microscopic count. Each of the inoculated samples including unaged controls was then incubated for 3-1/2 hours at 37°C. At the end of that period a bacterial count was made of each of the samples. The data summarized in Table 13 indicate that all of the aged samples of broth except the one stored in contact with EPR permitted the same extent of bacterial growth as an unaged control (i.e., unaged dextrose broth inoculated with E. coli). Apparently some component in EPR which leached into the nutrient inhibited the growth of the E. coli on incubation. It is to be concluded that containers for storing nutrient broth should not contain EPR.

Table 13

DEGRADATION OF DEXTROSE BROTH
WHEN AGED IN VARIOUS MATERIALS

Condition: 2.46×10^6 E. coli suspended in 5.5 ml of broth and incubated for 3-1/2 hours at 37°C. Broth had previously been aged for 8 weeks in contact with the materials as stated.

	E. coli	
Sample	Before Incubation	After Incubation
316 Stainless Steel	4.5×10^5/ml	2.3×10^7/ml
Ethylene Propylene	4.5×10^5/ml	4.3×10^5/ml
Viton B	4.5×10^5/ml	2.1×10^7/ml
PVC	4.5×10^5/ml	2.15×10^7/ml
Aged Nutrient (Control)	4.5×10^5/ml	2.24×10^7/ml
Unaged Nutrient (Control)	4.5×10^5/ml	2.4×10^7/ml

5.0 CONCLUSIONS AND RECOMMENDATIONS

In keeping with the program objectives the design, fabrication, and checkout of an Automatic Chemiluminescence Bacteria Detection System based on the filter-capsule approach have been completed. The instrument is capable of operating in a manual or automatic mode as desired. Some difficulties associated with capsule loading, transporter indexing, and station cylinder retraction occasionally appeared but can be easily solved by improving the capsule assembly procedure. A problem of greater difficulty associated with nutrient sterility has not been demonstrated at this time; however, an ethylene-propylene bladder has been purchased which may allow wet autoclaving to adequately sterilize the container.

Sensitivity goals of the instrument (< 5 cells/ml viable, < 10 cells/ml total, both for 400-ml sample) have been exceeded. Tests were conducted at challenges of 4×10^3 E. coli total cycle (10 cells/ml for 400-ml sample) and 2×10^3 E. coli viable cycles (5 cells/ml for 400-ml sample) and were reliably demonstrated repeatedly with a minimum 50% increase in signal. Challenges of 8×10^2 E. coli total cycle (2 cells/ml for 400-ml sample) revealed significant increases in signal. More testing should be run to adequately determine the true threshold sensitivity of the instrument. Background levels of the system varied significantly due to nutrient/system contamination and/or water contamination, causing signal levels to vary; however, challenges of 2 to 4×10^3 E. coli were always detectable. In addition the modification of electronics to an integrating amplifier signal processing system may improve the resolution of the data.

Other program objectives carried out include laboratory support studies in (a) capsule development, (b) support studies for development of the read station, (c) container and reagent feed system development and (d) reagent compatibility and shelf-life studies. Laboratory studies and tests were primarily performed to aid in determining design parameters and choise of compatible materials to be used in the instrument.

APPENDIX A

SYSTEM OPERATION PROCEDURE

1. Basic requirements for system operation:

   100 psi shop air
   110V, 60 cycle power
   0-5 VDC strip chart recorder with 25 cm/hr (10"/hr) paper speed

2. Prior to system startup the following must be accomplished:

   a. Containers must be filled as per the filling procedure outlined in Appendix B or C, with reagents prepared as outlined in Appendix D.

   b. Capsules must be cleaned, assembled and pretreated as described in Appendix E. Should more exact numbers be desired for tests, the capsules should be pretreated at the read station while recording capsule contamination levels taking the best capsules for tests.

   c. Water must be prepared as described in Appendix D for water blanks as well as bacterial suspensions.

3. Automatic Operation

   a. Install pretreated capsules in loader.

   b. Open all valves on the containers and in the fluid flow lines to pumping system.

   c. Set container pressure to 25 psig.

   d. Pump pressure should be set at 90 psig.

   e. Verify that waste carriers have waste capsules installed.

   f. Index the transporter system to load a capsule into any test carrier. Load capsule (may be old capsule for first run) into carrier by using the manual control box.

   g. Relocate transporter system to index the capsule loaded at the nutrient station.

   h. Start the nutrient cycle by engaging the nutrient switch on the instrument control panel. While the nutrient cycle is in process, the auto/man

switch may be switched to the auto position to put the system in automatic operation.

i. When taking the instrument out of automatic cycle, it is best to have the instrument panel switch activated for the station which is in process. For example, if the instrument is in automatic cycle and the read station is in process, switch the front panel switch marked "Read" to the up position and then switch the auto/man. switch to the manual position. The read process will then complete its cycle and stop.

4. Manual Operation

Manual operation may be performed only when the auto/man. switch is in the manual position. Manual operation is performed by the manual control box and allows every electrical operation to be initiated independently with lights indicating the function being operated. Lights on the control box operate in either the automatic or manual mode of operation. Care must be taken when using the control box since a test carrier need not be in position for the functions to operate.

5. Station-Automatic Operation

Switches on the instrument control panel allow any station to go through its automatic cycle provided a test carrier is in the proper location and auto/man. switch is in the manual position. Switches on the control box may be operated simultaneously and will override the station-automatic operation.

6. Total/Viable Switch

The switch on the instrument control panel marked total/viable only controls the operation of the two-hour incubation. When the switch is in the total position, the incubation cycle is bypassed.

7. Wash Switch

The switch marked wash provides the flushing of the nutrient station with urea and water into the waste capsule. When running only total cycles, the wash need not be performed.

8. System Shutdown

a. For overnight shutdown, the nutrient system should be flushed with 50 cc of 4 molar urea from just downstream of the nutrient container through to the needle. This is accomplished by the following:

(1) Locate a waste capsule at station 2.

(2) On the control box, engage cyl. 2, waste pump, and SOV 2.

(3) Activate the nutrient pump until displaced.

(4) Deactivate nutrient pump and refill with urea by applying pressure with 50 cc urea syringe (connected at V_3 as shown in the fill procedure, Appendix B).

(5) Repeat 3 and 4, above, until 50 cc syringe is empty.

b. Place all four-way valves on containers to blocked position.

c. Vent containers.

d. Shut off power switch.

e. For shutdown longer than a week, the nutrient should be removed from its container and refilled with 4 molar urea.

APPENDIX B

"FILL-IN-PLACE" PROCEDURE

The reagent feed containers are pressurized and designed to feed liquids to the pumping system. The containers for the urea, water, and nutrient are of the rolling diaphragm type (a Viton B bladder separates the air and liquid phases, see Figure B-1) whereas a teflon bellows type is utilized for storing the luminol and hydrogen peroxide reagents.

Prior to installing reagent containers for the first time, the entire liquid flow system should be sterilized by flushing with 4 M Urea followed by sterile distilled water. These flushing liquids will be introduced into the system via the three-way valve, V_4. Each of the containers, which have been previously sterilized by either ethylene oxide or autoclaving are then connected to the system. The interconnecting line between V_3 and V_4 in Figure B-1 is then sterilized by flushing with 4 M Urea and sterile distilled water.

For filling the container "in situ" (Figure B-1), the container must first e evacuated on the liquid side of the bladder through a four-way valve (V_1). The latter is able to connect the liquid side of the container to any one of three ports (i.e., vacuum, fill or pump) as desired.

The filling procedure would be as follows:

1. With V_2 vented to the atmosphere, switch V_1 to the vacuum port and evacuate the liquid side of the container, collapsing the bladder.

2. Switch V_1 to Fill port.

3. Sterile reagent contained in a suitable container* is connected to the Fill port through a connectingtube and a sterile hypodermic needle. The latter pierces a rubber septum in the Fill port. Before piercing the rubber septum, a cellulose filter disc saturated with alcohol is placed in direct contact with the rubber septum. The sterile needle, enclosed in a close fitting rubber sleeve, is pressed up against the cellulose disc puncturing the sleeve and the rubber septum.

*Recommended containers are outlined in notes of Appendix C.

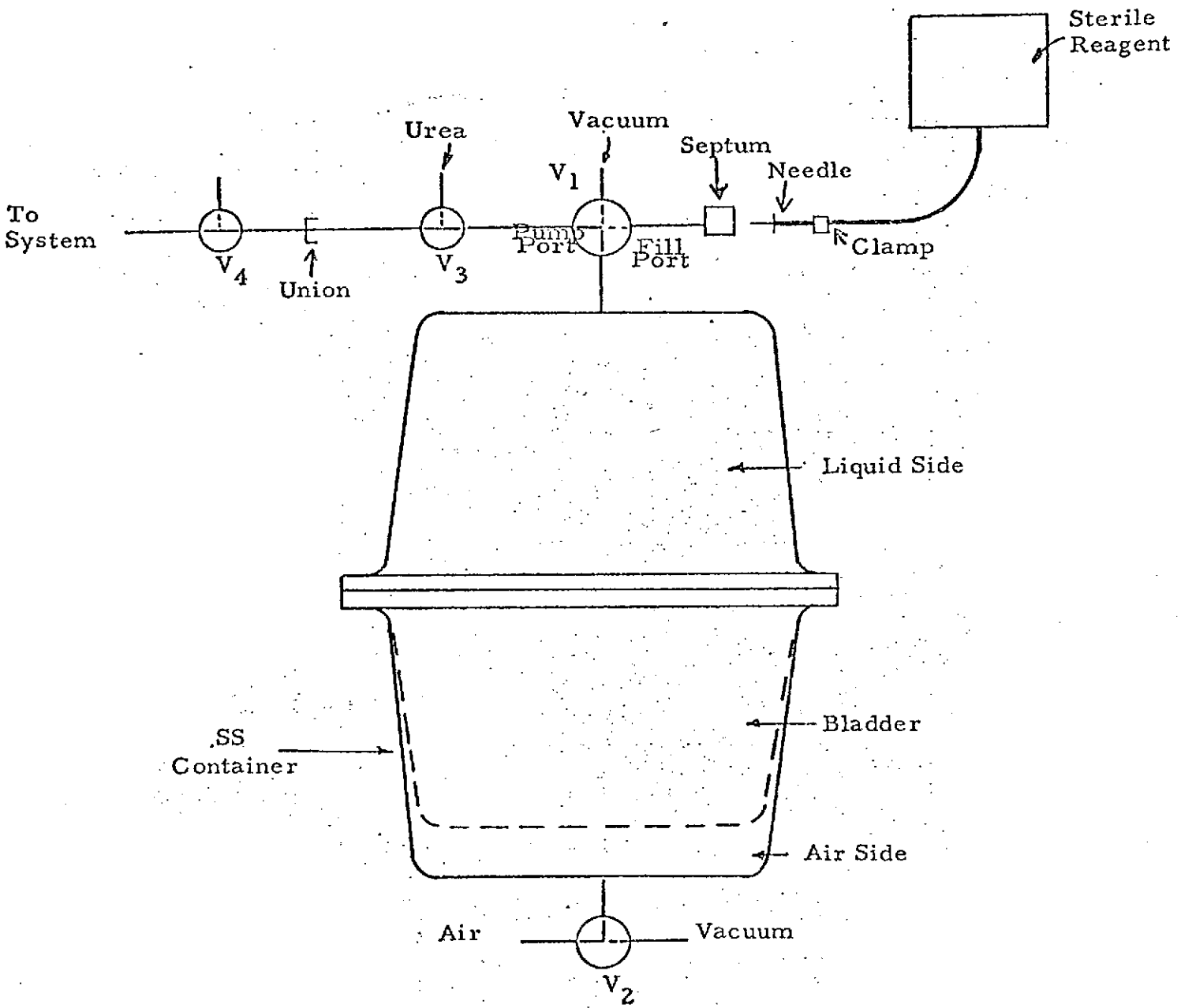
4. With V_2 vented to the atmosphere, the liquid is fed into the container under pressure (a slight vacuum applied through V_2 may be required to aid in filling).

5. Withdraw needle from septum.

6. Switch V_1 to Pump port and pressurize the air side of the bladder through V_2. The system is now ready for operation.

7. For refilling the container, if sterilization of the latter is not required, see Appendix C. The rubber septum* is reusable and sterilizable. These septa should be good for at least a dozen punctures before requiring replacement.

*Microsep F-138 Septa from Hamilton Company, Reno, Nevada.



Rolling Diaphragm Reagent Container

Figure B-1

APPENDIX C

CONTAINER REFILL PROCEDURE

Using an appropriately sized sterile-evacuated plasma flask* from Cutter Laboratories, remove outer seal and rubber stopper. Fill with appropriate reagent and reinstall rubber stopper. For nutrient, the autoclaving procedure outlined in Appendix D, Section D, must be followed at this point.

To transfer sterile liquid contents to reagent container on the system, a Saftiset transfer tube** is used. Swab septum stopper of plasma flask with alcohol and immediately insert sterile wide bore needle into septum. Swab inlet septum on system container with alcohol and insert sterile 20-gauge needle. Open interconnecting clamp between the flask and the reagent container to start liquid flow.

To refill or fill the H_2O_2 and luminol container, a 50-ml, disposable syringe with a 20-gauge needle is used to add liquid through the four-way valve septum. After syringe is bottomed-out, release 10-ml back into syringe to receive excessive head pressure.

*690 ml - No. 8161 - 0767-29, Cutter Labs, for nutrient.
2000 ml - No. 8161 - 0766-31, Cutter Labs, for urea and water.

**Cutter Laboratories, No. 8161-0860-02. This is a plastic tube with a 20-gauge hypodermic needle on one end and a wide bore needle (with an integral air filter) on other end. Supplied sterile with removable plastic caps on both ends.

APPENDIX D

REAGENT PREPARATION

A. DISTILLED WATER

The "filtered distilled water" used in bacterial suspensions and reagents is prepared fresh daily as follows:

Bottled distilled water is redistilled* (glass still) and distillate collected in polypropylene bottles. The latter are sterilized by autoclaving once a week and rinsed with two 250-ml volumes of filtered distilled water prior to use.

The collected distillate is then filtered through a 0.22 μ pore size Millipore filter.** The filtered, distilled water required for use is taken directly from the filter flask.

B. LUMINOL - H_2O_2 REAGENT

1. Stock Luminol Solution

a. 60.0 gms sodium hydroxide ("Baker Analyzed" Pellet Reagent) are dissolved in a liter of filtered, distilled water and allowed to cool to ambient temperature.

b. 15.0 gms disodium ethylenedinitrilotetracetate dihydrate ("Baker Analyzed" Reagent Powder) are dissolved in above alkaline solution.

c. Dissolve 1.00 gm of luminol (3-aminophthalhydrazide, Aldrich Chemical) in above. Let solution stand at ambient temperature overnight before use.

d. The stock luminol solution may be stored in an unpigmented polyethylene or polypropylene container. The solution has a shelf

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*Glass still is treated (1/2 hour distillation) with concentrated (~ 35%) HCl, followed by repeated distilled water flushes until distillate has a pH = 6.0 + 0.5. This treatment is performed once a week to remove mold and bacterial slime.

**About 500 ml of distilled water is passed through the Millipore filter prior to use to remove finishing agent from filter.

life at ambient temperature of at least 6 months. Freezing does not affect the luminol solution; however, elevated temperatures accelerate the decomposition (with loss in sensitivity). For maximum shelf life, storage temperatures should not exceed 95°F.

2. Luminol Reagent

Dilute 16.30 ml of stock luminol to 100 ml volume with filtered, distilled water. Use this solution to charge reagent container.

3. Hydrogen Peroxide Reagent

Dilute 11.84 ml of 3.0% H_2O_2 ("Baker Analyzed" Reagent) to 100-ml volume with filtered, distilled water. Use this solution to charge reagent container.

4. Luminol - H_2O_2 Reagent

Each of the luminol and H_2O_2 reagents are injected into the system in a 1:1 volume ratio.

C. 4 M UREA SOLUTION

Reagent grade ("Baker Analyzed") Urea is dissolved in glass-distilled water to a final concentration of 4 Molar. The solution is filtered through a 0.1 μ Ultripor filter and then passed through a mixed bed ion exchange column (equal quantities of Dowax AG 50W - X8 and Dowax AG1 - X8, 200-400 mesh, 200-ml bed volume). The pH of the effluent from the column is adjusted to pH 7.0 to 7.5 with hydrochloric acid and then filtered through a 0.2 μ (prewashed) Millipore prior to use.

The 4 M Urea may be stored in a glass, polyethylene or polypropylene container prior to charging the urea container. The solution is stable for at least 7 weeks at temperatures up to 37°C.

To determine whether significant deterioration of a Urea solution has occurred on standing, the baseline signals obtained with the prescribed processing sequence are compared for aged and unaged Urea solutions. If significant decomposition has occurred, the aged Urea solution would be less effective in removing nutrient and so produce higher baseline values on reaction with luminol-H_2O_2.

D. DEXTROSE BROTH

1. Add 23 gms of Dextrose broth (Difco) to one liter of distilled water.

2. Heat and stir until dissolution is complete.

3. While still warm, filter through the following filter sequence:

   a. Four No. 50 Whatmans (4 layers)
   b. 0.8 Millipore AAWP
   c. 0.4 Millipore HAWP
   d. 0.22 Millipore GSWP

4. Dispense into plasma bottles; replace bottle stopper using glycerine as a stopper lubricant; filter-vent the plasma bottle.

5. Autoclave @ 15 psi for 15 minutes.

6. Liquid-cool down and let cool to room temperature in the autoclave.

7. Remove the filter vent and cap-seal the vent area with RTV silicone rubber.

8. Let the RTV cure for one hour.

9. Invert the plasma bottle, shake well, and incubate @ 32°C for 16 hours.

10. If the broth remains clear, use for test.

E. PREPARATION OF BACTERIAL SUSPENSION

1. Inoculate TSA (Tryptic soy agar) slants with Escherichia coli and incubate 16 hours at 37°C (overnight).

2. Using a sterile 1.0 ml pipette, touch the smear of the TSA slant growth with the pipette tip. Withdraw the tip and immerse it in a sterile container containing 100 ml of sterile filtered water at room temperature. Cap the flask and shake well.

3. Dilute the aforementioned suspension one to 10 final.

4. Do a total count using a 1-ml sample volume, i.e., Pink RL Technique.

5. Dilute empirically for test. Store the stock suspension in the refrigerator at 11°C.

APPENDIX E

CAPSULE ASSEMBLY PROCEDURE

1. Anneal capsule lid and body for 2 and 6 hours, respectively, at 82°C (wrapped in aluminum foil).

2. After removing from oven - place immediately between 1" thick styrofoam pads to ensure slow cooling to ambient.

3. Sonicate annealed lid and body in a Hemo-sol solution for 15 minutes.

4. Wash plastic components thoroughly with Super-Q distilled water.

5. Soak plastic components for 30 minutes in 4 M Urea.

6. Wash plastic components in filtered, distilled water.

7. Place plastic components in a partially open Petri dish and allow to air dry.

8. The filter membrane, O-ring, and Viton septum were washed with 4 M Urea and filtered, distilled water as indicated by steps 5 and 6, above, and air dried (as in step 7).

9. Prepare PS-30 glue (IPS) as follows:

   a. Mix Part "A" 4.75 grams to Part "B" 0.25 grams.
   b. Mix glue for about 2 minutes.
   c. Let stand until bubbles leave and mix takes on a yellow hue.

10. Apply layer of PS-30 glue (IPS) to the surface of the capsule body with the end of a glass pipette (1 ml).

11. After completely covering the surface of the capsule body with the adhesive, let it set for 4 to 5 minutes to allow adhesive to settle out and form an even film.

12. Drop filter-membrane (Acropor AN 450) and O-ring in place; line up lid with body and drop in place.

13. Place capsule in clamping fixture for 2 hours.

14. Remove capsule and trim excess dried glue with a file, clean guide pin holes with #9 drill, and insert septum.

15. Inject 5 ml of Premix into capsule using a hand syringe. Allow to sit 10 minutes. Flush 20 ml of filtered, distilled water through capsule. Repeat above cycle two more times.

16. Place capsule in instrument and check for luminescence at readout station (after injecting luminol - H_2O_2 mixture). Equilibrium signal after 4 minutes should not exceed 8 volts (on analog readout).

17. Flush capsule with filtered, distilled water.

18. Blow filtered air through capsule to remove bulk of water.

19. Store capsule in a dessicator over P_2O_5 (at ambient) until ready for placing in loader.

APPENDIX F

CASSETTE BACTERIA DETECTION SYSTEM
MASTER TEST PLAN
Contract NAS 9-13256

A. Mechanical and Electrical Checkout

Prior to an evaluation of its sensitivity and reliability, the assembled system will be subjected to a mechanical and electrical checkout to assess its functionability. Features which will be examined in this preliminary phase include:

1. Manual and Automatic Operational Modes

The proper functioning of each of the stations on actuation of the manual control switches will be determined. Particular emphasis will be placed on the proper loading and transport of the capsules, station alignment, fluid transport and the light-sealing efficiency at the last station. The ability of the incubator to maintain the proper temperature control will also be assessed.

The ability of the system to maintain the prescribed sequence logic in the automatic mode during the total and viable cycles will be determined.

More specifically, the following will be evaluated:

a. Loading Station

The ease with which capsules can be stacked into the loading container will be noted, as will be the ease with which the container can be installed and removed from the system. Loading of the capsule onto the carrier must be smooth without hangup; in the automatic mode, the loading logic should insure that indexing to the next station does not occur until a capsule is properly loaded in the carrier.

b. Sample Station and Plunger Assembly

Tests will be performed to insure that the sample pump delivers a consistent water sample by checking for a constant flow rate over a prescribed pressure range (i.e. 15 to 40 psi).

c. Incubator

The incubator will be tested by use of a dummy capsule properly instrumented to insure that the proper filter temperature (of 37°C) is reached and maintained as soon as practical after arrival of the capsule at the incubator. A standard capsule will be modified to contain either a thermometer or thermocouple to measure the temperature at the filter. The capsule will be filled with liquid during test. Tests will be run to determine the temperature profile that will be required in the heating block (in contact with the capsule in the incubator) to bring the capsule to temperature rapidly and maintain it during the two hour incubation.

The ability of the capsule to be incubated for two hours without drying up in the incubator will also be evaluated by visual means.

d. Reaction Readout Station

Tests will be conducted to determine that the light sealing is adequate and consistent during reaction and readout. With voltage on to the PMT, and the capsule in position, the extent of light leakage should preferably be less than two volts (as measured on the strip chart recorder).

The signal processing electronics (i.e., analog and net signal readouts) will be checked by recording them on a strip chart recorder at different signal levels to determine that the recorded net signal is actually the difference between the maximum signal and the reagent blank signal. Bacterial challenges (E. coli) reacted with luminol-H_2O_2 reagent at the PMT will be used to generate the signals at the various levels.

e. Unload Station

The unloader must properly eject the capsule from the carrier into the holding container. Capsules should be readily removable from the holding container

f. Reagent Pumps and Check Valves

The liquid pumps will be operated to determine the optimum air pressure requirements for actuation.

To keep bubbles from building up in the capsule, liquid pressures will be checked down stream of the check valves to insure that the bubble point in the capsule is being exceeded.

Pumps will be adjusted to deliver the correct volume of reagent and checked periodically to see how well they maintain this volume.

g. Waste Pump System

During the initial testing phase, pressures will be checked in the waste lines to insure proper flow and minimize leakage at the O-rings.

h. Transporter/Drive System

The drive system will be adjusted to assure adequate drive power and proper alignment at the stations. The extent of capsule misalignment, which can be tolerated before the guide pins fail to engage the capsule, will be determined.

i. Malfunction Safeguards

The system will be made to malfunction to determine if the built-in safeguards are functioning properly, for example;

(1) the capsule loading station will be permitted to empty to determine if the system shuts down at the next loading command,

(2) fluid containers will be allowed to run dry to determine if operation ceases,

(3) the electrical power will be momentarily interrupted to determine if the system remains off after power is restored,

(4) the electrical power will be interrupted part way through the index cycle to determine if carrier movement is completed,

(5) the manual index stop will be tested to see that it provides proper manual alignment.

B. Feasibility Testing of Fill Procedure

The feasibility of the "Fill-In-Place" Procedure outlined in Appendix A will be examined. Apart from the possible problem of filling the container completely, the ability to prevent bacterial contamination of the liquid contents will have to be demonstrated. This would present a problem only for the nutrient or water containers, since the other liquids are good bacteriostats. Contamination would be reflected by abnormally high water-reagent blank signals.

C. Capsule Integrity and Protocol Acceptability Evaluation

After the assembled instrument has been subjected to a mechanical/electrical checkout, and feasibility of the fill procedure established, the assembled and pretreated capsule will then be evaluated (see Table1) for evidence of (1) plugging or leakage, (2) flow rate impairment and (3) evidence of contamination. With respect to the last item, by running a water-reagent blank with and without nutrient in the standard protocol, one could tell whether an observed high signal was due to an inadequate capsule pretreatment, or an inadequate sample processing protocol which failed to remove all of the nutrient from the capsule.

In terms of its effect on sensitivity, the delivered liquid volume is critical only for luminol-hydrogen peroxide reagent at the readout station. Hence, the effect of this parameter on sensitivity will be evaluated using 0.4, 0.6 and 0.8 ml delivered volumes of luminol-H_2O_2 reagent against a given bacterial (E. coli) challenge (in a total cycle only).

D. Sensitivity and Reliability

After capsule integrity and protocol acceptability have been established, the system will be tested for sensitivity and reliability with the total and viable cycles, as indicated in Tables 2 and 3.

E. Zero-G Capability

The final test involving Zero-G simulation (Table 4) will serve to demonstrate that the system can be operated in a Zero-G environment.

F. Testing Schedule

The proposed testing schedule is shown in Table 5.

If the system evaluation phase is completed ahead of schedule, additional tests will be performed to confirm the statistical reliability (i.e., repeatability at various detection levels) of the instrument.

TABLE 1

CAPSULE EVALUATION

1. Before Assembly

10% of batch* of unassembled parts to be checked for:

a. major diameter of capsule
b. thickness of compression ring in lid
c. diameter of guide pin holes
d. transmission of capsule window at 430 mu

2. After Assembly and Pretreatment (Prior to Loading)

a. General appearance (uniformity of glue line, etc.) (100% of batch).
b. Pump 50 ml of filtered distilled water thru capsules (sample pump set at 20 ml/min at 30 psi) and check for:
   (1) visual evidence of plugging or leakage (10% of batch),
   (2) flow rate thru capsule (with sample pump set at 20 ml/min at 30 psi) - (10% of batch),
   (3) water-reagent blank signal using total and viable cycles.‡ A high blank signal in the total cycle could be the result of interaction of residual nutrient which had not been washed out of the crevices, with the luminol-H_2O_2 reagent. The remedy in this instance would be a better capsule sealing and assembly procedure to eliminate the crevices. A high water blank in viable cycle could indicate a contaminated capsule or water supply.

3. Applicable data sheet attached. (Table 1A)

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* A batch here is defined as the number that might be subjected to the same glueing or pretreatment procedure.

‡ Total cycle run with and without nutrient (see Table 2).

TABLE 1A
DATA SHEET NO._____

DATE: _____

CAPSULE EVALUATION OPERATOR_____

A. Before Assembly (10% of batch)

1. Glueing and Assembly Procedure:

2. Dimensions and Trans.

a. Major diam. of capsule _____

b. Thickness of lid compression ring _____

c. Diam. of guide pin holes _____

d. Trans. of capsule window at 430 mu _____

B. After Assembly and Pretreatment (10% of batch)

1. Pretreatment:

2. Performance:

Pump 50 ml of filtered dist. H_2O thru capsules (sample pump set at 20 ml/min, 30 psi).

a. Plugging? _____

b. Leakage? _____

c. Flow rate thru filter _____

d. Water-reagent blank signal

- total cycle _____

- viable cycle _____

C. Comments:

TABLE 2
TOTAL CYCLE EVALUATION

Using conditions specified in Appendix B, perform the following tests with E. coli and S. Marcescens as the test organisms.

1. Detection Threshold

Establish the detection threshold for each organism using initially a concentration of 100 cells/ml (400 ml sample). Depending on the magnitude of the signal, select two other concentration points (above or below) which are set at least 1/2 log higher or lower. Make at least two determinations at each bacterial level.

Run water reagent blank at beginning and end of series each day.

2. Repeatability

Perform minimum of five runs at the lowest detection level and five runs at 10 times the lowest detection level. Run water-reagent blank at beginning and end of series each day. The net signal (i.e., signal on subtracting water blank from sample signal) will be determined for each bacterial level, and the standard deviation from the mean value derived. The standard deviation will be used to define the detectability and reliability at a given bacterial level.

3. Applicable Data Sheet Attached. (Table 2A)

TABLE 2A
SYSTEM EVALUATION

Date _____

Data Sheet No. _____

Operator _____

Run No.	Total or Viable	Sample Size	Cells/ml	Total Chall.	Background (above zero)	H_2O Blank (above zero)	Signal (above zero)	Net Signal (volts)	Comments

Test Conditions

PMT _____

Incubation Temp. _____

Incubation Time _____

Operational Mode _____

	Reagents			
	Nutr.	Urea	Luminol	H_2O_2
Batch No.	____	____	_____	_____
Deliv. Volume	____	____	_____	_____
Other	____	____	_____	_____

TABLE 3

VIABLE CYCLE EVALUATION

Using conditions specified in Appendix B, perform the following tests with E. Coli and S. Marcescens as the test organisms.

1. At the minimum detection level for test organism, run unincubated and (2 hr) incubated water blank, unincubated and (2 hr) incubated bacterial sample. Make minimum of five (5) replicate determinations for each organism. The net signal (i.e., signal on subtracting water blank from sample signal) will be determined for each bacterial level, and the standard deviation from the mean value derived.

2. Applicable data sheet shown in Table 2A.

TABLE 4

ZERO-G SIMULATION

1. Perform following using E. Coli as the test organism:

   (a) Total cycle (water blank and unincubated sample)

   (b) Viable cycle (water blank and sample incubated 2 hours)

   Preferably (a) and (b) are to be performed on a single day with the instrument either upside-down on in some other orientation which is determined to be more representative of zero-G simulation. The above is to be performed in duplicate, time permitting.

2. Applicable data sheet shown in Table 2A.

Table 5

TESTING SCHEDULE

Task	Description	Expected Completion Date
A	Mechanical and Electrical Checkout of Assembled System	Oct. 26, 1973
B	Fill Procedure Evaluation	Nov. 9, 1973
C	Capsule Integrity and Protocol Acceptability Evaluation	Nov. 26, 1973
D	Sensitivity and Reliability	
	(1) Total Cycle Evaluation	Dec. 13, 1973
	(2) Viable Cycle Evaluation	Jan. 10, 1974
E	Zero-G Capability	Jan. 18, 1974

APPENDIX A OF MASTER TEST PLAN

"FILL-IN-PLACE" PROCEDURE

The reagent feed containers are pressurized and designed to feed liquids to the pumping system. The containers for the urea, water and nutrient will be of the rolling diaphragm type (a Viton B bladder separates the air and liquid phases, see Figure 1) whereas a teflon bellows type will be utilized for storing the luminol and hydrogen peroxide reagents.

Prior to installing reagent containers for the first time, the entire liquid flow system will be sterilized by flushing with 4M Urea followed by sterile distilled water. These flushing liquids will be introduced into the system via the 3-way valve V_4. Each of the containers, which had been previously sterilized either by ethylene oxide or autoclaving are then connected to the system. The interconnecting line between V_3 and V_4 in Figure 1 is then sterilized by flushing with 4M Urea and sterile distilled water.

For filling the container "in situ" (Figure 1) the container must first be evacuated on the liquid side of the bladder through a 4-way valve (V_1). The latter is able to connect the liquid side of the container to any one of three ports (i.e., vacuum, fill or pump) as desired.

The filling procedure would be as follows:

1. With V_2 vented to the atmosphere, switch V_1 to the vacuum port and evacuate the liquid side of the container, collapsing the bladder.

2. Switch V_1 to Fill port.

3. Sterile reagent contained in a suitable container* is connected to the Fill port through a connecting tube and a sterile hypodermic needle. The latter pierces a rubber septum in the Fill port. Before piercing the rubber septum, a cellulose filter disc saturated with alcohol is placed in direct contact with the rubber septum. The sterile needle, enclosed in a close fitting rubber sleeve, is pressed up against the cellulose disc puncturing the sleeve and the rubber septum.

* Container with connecting tubing and reagent (i.e. water or nutrient) are sterilized by autoclaving using standard sterilization procedures.

4. With V_2 vented to the atmosphere, the liquid is fed into the container under pressure (a slight vacuum applied through V_2 may be required to aid in filling).

5. Withdraw needle from septum.

6. Switch V_1 to Pump port and pressurize the air side of the bladder through V_2. The system is now ready for operation.

7. For refilling the container, if sterilization of the latter is not required, repeat steps 1 through 6 above. The rubber septum* is reusable and sterilizable. These septa should be good for at least a dozen punctures before requiring replacement.

*Microsep F-138 Septa from Hamilton Co. (Reno, Nevada)

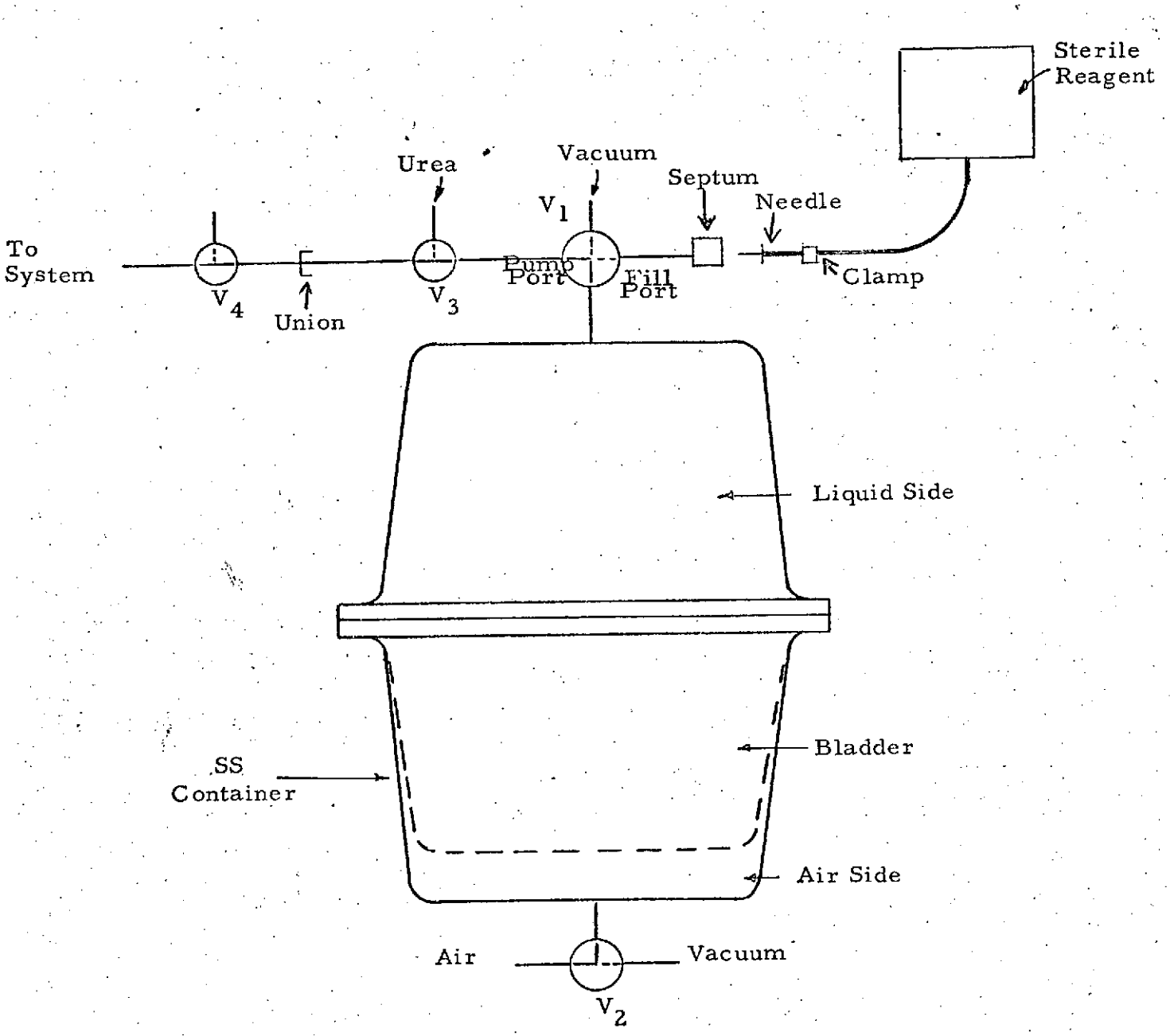

Figure 1. Rolling Diaphragm Reagent Container

APPENDIX B OF MASTER TEST PLAN

PRELIMINARY OPERATING TEST CONDITIONS

Station	Function	Flow Rate ml/min	Total Volume* Delivered, ml	Duration, Min Total	Duration, Min Viable
Load	Load Capsule	-	-	~1/4	~1/4
1	Sample Concentration	20	400	20	20
2	Nutrient Added	-	5.5	~1/2	~1/2
	Urea Cleanup (Second Capsule)	-	4.0	~1/2	~1/2
	Water Cleanup (Second Capsule)	-	4.0	~1/2	~1/2
3	Incubation at 37°C (± 1°C)	-	-	0	120
4	Urea Wash		6.0	~1/2	~1/2
5	Premix Reaction/ Readout**	-	0.6	8‡	8‡
	Water Wash	-	4.0	~1/2	~1/2
Unload	Unload Capsule	-	-	~1/4	~1/4
			Total Time, min.	~31	~151

* Except for the sample volume for which a 5% maximum in delivered volume is permitted, up to a 10% variation is allowed for the other liquid volumes.

** PMT voltage - 1180 volts (± 25 volts).

‡ Premix reagent flow on for maximum of 10 seconds; flow then ceases and reaction permitted to proceed for remainder of 10 minutes; preparation of reagents is described in Appendix C.

2. Luminol Reagent

Dilute 16.30 ml of stock luminol to 100 ml volume with filtered distilled water. Use this solution to charge reagent container.

3. Hydrogen Perioxide Reagent

Dilute 11.84 ml of 3.0% H_2O_2 ("Baker Analyzed" Reagent) to 100 ml volume with filtered distilled water. Use this solution to charge reagent container.

4. Luminol - H_2O_2 Reagent

Each of the luminol and H_2O_2 reagents are injected into the system in a 1:1 volume ratio.

C. 4M UREA SOLUTION

Reagent grade ("Baker Analyzed") Urea is dissolved in glass-distilled water to a final concentration of 4 Molar. The solution is filtered through a 0.1 u Ultripor filter and then passed through a mixed bed ion exchange column (equal quantities of Dowax AG 50W - X8 and Dowax AG1 - X8, 200-400 mesh, 200 ml bed volume). The pH of the effluent from the column is adjusted to pH 7.0 to 7.5 with hydrochloric acid and then filtered through a 0.2 u (pre-washed*) Millipore prior to use.

The 4M Urea may be stored in a glass, polyethylene or polypropylene container prior to charging the Urea container. The solution is stable for at least 7 weeks at temperatures up to 37°C.

To determine whether significant deterioration of a Urea solution has occurred on standing, the baseline signals obtained with the prescribed processing sequence are compared for aged and unaged Urea solutions. If significant decomposition has occurred, the aged Urea solution would be less effective in removing nutrient and so produce higher baseline values on reaction with luminol -H_2O_2.

D. DEXTROSE BROTH

1. Add 23.0 grams of Difco Dextrose broth to 1 (one) liter of freshly distilled water.

2. Heat and stir until dissolution is complete.

3. Filter solution progressively through Nos. 3, 5 and 50 Whatman filters followed by filtration through a 0.8 u, 0.45 u and 0.22 u membrane filters.

4. Use above solution to charge reagent container.

E. PREPARATION OF BACTERIAL SUSPENSION

Bacteria (E. Coli or S. Marcescens) are 16 hour cultures grown on trypticase soy agar at 37°C. Bacteria are harvested by adding 3 to 4 ml filtered distilled water to make up the stock bacterial suspension. Final dilutions are made up in filtered distilled water.

"Total" (viable + non-viable) bacterial counts are made by direct microscopic count after prestaining the organism with an AMB protein-specific dye.

"Viable" counts are made at the time a sample is taken for processing of the viable cycle on the water monitor. The standard pour plate procedure is used for obtaining a viable count.

APPENDIX C OF MASTER TEST PLAN

REAGENT PREPARATION

A. DISTILLED WATER

The "filtered distilled water" used in bacterial suspensions and reagents is prepared fresh daily as follows:

Bottled distilled water is redistilled* (glass still) and distillate collected in polyproplyene bottles. The latter are sterilized by autoclaving once a week and rinsed with (2) 250 ml volumes of filtered distilled water prior to use.

The collected distillate is then filtered through a 0.22 μ pore size Millipore filter**. The filtered distilled water required for use is taken directly from the filter flask.

B. LUMINOL - H_2O_2 REAGENT

1. Stock Luminol Solution

a. 60.0 gms sodium hydroxide ("Baker Analyzed" Pellet Reagent) are dissolved in a liter of filtered distilled water and allowed to cool to ambient temperature.

b. 15.0 gms disodium ethylenedinitrilotetracetate dihydrate ("Baker Analyzed" Reagent Powder) are dissolved in above alkaline solution.

c. Dissolve 1.00 gm of luminol (3-aminophthalhydrazide, Aldrich Chemical) in above. Let solution stand at ambient temperature overnight before use.

d. The stock luminol solution may be stored in an unpigmented polyethylene or polypropylene container. The solution has a shelf life at ambient temperature of at least 6 months. Freezing does not affect the luminol solution; however, elevated temperatures accelerate the decomposition (with loss in sensitivity). For maximum shelf life, storage temperatures should not exceed 95°F.

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* Glass still is treated (1/2 hour distillation) with concentrated ($\sim$ 35%) HCl, followed by repeated distilled water flushes until distillate has a pH $\simeq$ 6.0$\pm$0.5. This treatment is performed once a week to remove mold and bacterial slime.

** About 500 ml of distilled water is passed through the Millipore filter prior to use to remove finishing agent from filter.